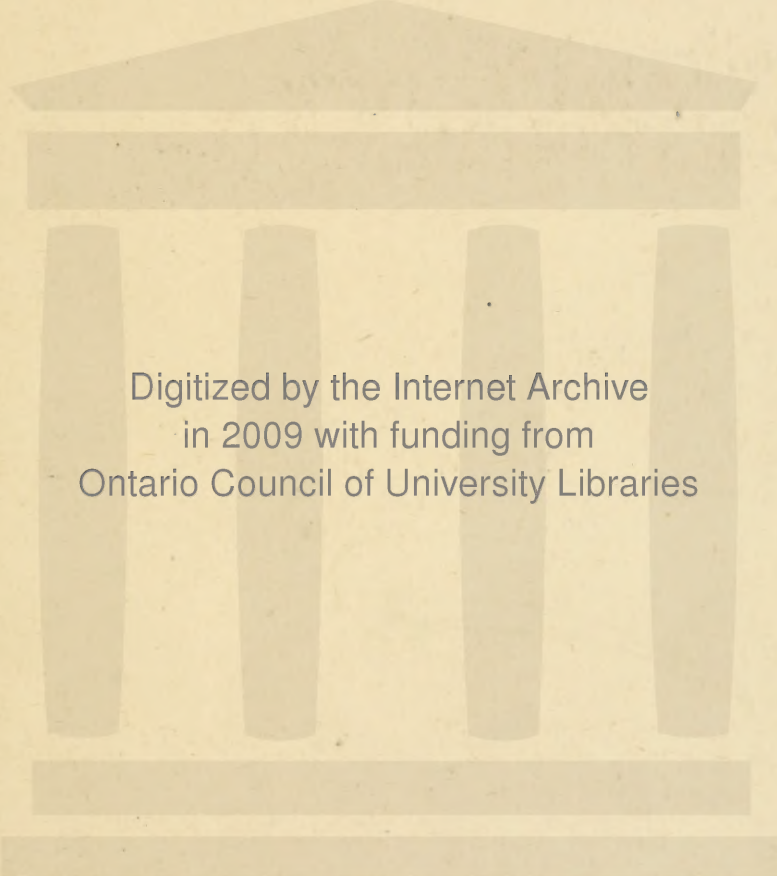


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# BIO-CHEMICAL JOURNAL

EDITED BY

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AND

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## ON THE FORMATION OF LACTOSE

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*(Received October 16th, 1906)*

The following note contains an account of certain experiments which were undertaken to test Paul Bert's hypothesis regarding the formation of lactose from glucose.

Bert's first experiment was upon a guinea-pig, in whose mammary glands he searched for an intermediate substance out of which it would be possible to form lactose in an appreciable quantity, but he failed to discover any such substance. He next proceeded to remove the mammary glands from two goats which were subsequently allowed to become pregnant, and he tested the urine of these animals to see if he could find a reducing substance both during the period of pregnancy and for a short time after parturition. No such agent could be found in the urine of either of the goats during pregnancy, but in one of them for about nine days after the birth of the kid a substance which markedly reduced cupric sulphate was discovered in considerable quantity, while in the case of the other goat the effect produced was similar though less pronounced. Bert concluded that the reducing substance was glucose, but it does not appear that he employed any further test to differentiate it from other reducing agents. He supposed further that the glucose in the urine represented glucose which in normal animals would have been converted into lactose in the mammary glands.

Bert's experiments were repeated by Moore and Parker, who first examined normal goat's urine, and found that it gave a feeble reduction with Fehling's and Almen's tests, but not sufficient to indicate the presence of an appreciable quantity of sugar. The mammary glands of two pregnant goats were subsequently removed. Parturition occurred in due course. In the one case no change

whatever could be detected in the urine after parturition, and in the other only a very slight increase in the reducing power of the urine was discernible for two days, disappearing on the third day. No result was obtained with the phenylhydrazine test, and in the opinion of the investigators 'no reducing *sugar* was present, but only traces of a substance with feeble reducing power.' These results, therefore, were opposed to those of Bert, and the authors conclude that the complete process of lactose formation takes place in the cells of the mammary glands.

The question of lactose formation has been recently reopened by Porcher, who does not appear to have been aware of the experiments of Moore and Parker. Like the latter, Porcher also operated on a goat, repeating Bert's experiment. After parturition, which is said to have taken place normally, an 'intense glycosuria' occurred. The phenylhydrazine test showed that the sugar was glucose, and not lactose or maltose. Porcher also removed the mammary glands from four goats and one cow during lactation, and for a few hours after the operation obtained marked glycosuria. As a result of these experiments he concludes that the truth of Bert's theory is established. 'Il paraît donc irréfutablement démontré que la mamelle est l'organe producteur du lactose et que, pour l'élaboration de ce dernier sucre, elle en emprunte les matériaux au glucose que lui apporte le sang.' 'Il n'est pas niable que l'activité de la glande hépatique doive être plus grande pendant la lactation, puisque cet organe, dont un des rôles est de déverser le sucre dans le sang, devra en outre faire face, en vue de la sécrétion lactée, à la mise en liberté du glucose qui sera ultérieurement transformé en lactose.' (Porcher, 1906.)

For the purpose of our own experiments we made use of guinea-pigs, which differ from most other rodents in having only two mammary glands and teats situated one on each side of the abdomen. We removed the mammary glands from ten animals, only one of which was pregnant at the time of the operation. In some instances a single transverse incision from teat to teat was made across the skin of the abdomen, and each gland was dissected away from the skin and underlying tissue; in other cases two cuts were made,

one on each side, an uninterrupted bridge of skin being left between the two incisions while the mammary tissue was removed as in the former cases. The teats were also removed along with a small piece of skin. The edges of the cut skin were afterwards sewn together, and the wounds allowed to heal, a process which usually occurred very rapidly. Three of the guinea-pigs unfortunately died either from disease or accident some time after the wounds had completely healed. In each of these animals no trace of mammary tissue could be found post mortem. Considerable difficulty was experienced in inducing the guinea-pigs to breed after the removal of the glands, some of them failing to do so although kept for over eight months in company with males, and in an apparently perfectly healthy condition. Four guinea-pigs, however, eventually became pregnant. The following is an account of these four experiments:—

*Experiment I.*—The mammary glands were removed from a large rough-haired guinea-pig in the beginning of December. At the end of February the animal seemed to be pregnant.<sup>1</sup> The urine was tested and no sugar or albumen was found to be present. On April 7th one young one was born prematurely and found dead. On April 8th the urine collected during the previous night was tested with Fehling's solution, which turned a greenish-yellow colour on boiling for three minutes. No sugar could be detected with the phenylhydrazine test. On April 9th Fehling's solution gave no reduction whatever. This guinea-pig was again put beside a male and on July 30th was a second time observed to be palpably pregnant. Parturition did not occur until August 24th, when three young ones were born apparently at full time. The urine was tested for several days subsequently when no trace of sugar could be detected either by Fehling's test or by the phenylhydrazine test. The three young ones all survived, feeding for themselves in spite of the fact that the mother was unable to suckle them.

*Experiment II.*—The glands were removed from a small rough guinea-pig early in December. At the beginning of February it was observed to be pregnant. On February 21st the guinea-pig appeared to have aborted while in company with another guinea-pig but no foetus could be found, having probably been eaten. The urine was then examined and found by Fehling's and the phenylhydrazine tests to contain glucose in perceptible quantities. A small amount of albumen was also found to be present. The animal appeared to be ill and on February 28th died. The post mortem showed that the animal had died from the effects of tubercle on the liver and intestinal lymphatic glands. Signs of recent abortion were also noted. It is evident that this experiment was quite inconclusive.

1. The period of gestation in the guinea-pig is from sixty-three to sixty-six days.



*Experiment III.*—The mammary glands were removed from a smooth-haired guinea-pig early in March. On July 10th it had three young. The animal was immediately placed in the metabolism cage and the urine of twenty-four hours collected. The urine was then tested by Fehling's and Nylander's solutions. No trace of sugar could be detected. Also Dr. W. Cramer kindly tested with the polarimeter and found no indication of sugar. On July 12th (*i.e.*, next day) the urine was again tested with Fehling's solution which again gave no reduction. The same result was obtained next day.

*Experiment IV.*—The mammary glands were removed from a guinea-pig in an advanced stage of pregnancy on June 16th. Four young ones were born on June 20th. The urine was collected for the next twenty-four hours and examined on June 21st. Fehling's solution gave a yellowish green precipitate on prolonged boiling, but the phenylhydrazine test was negative. No albumen was present. The same result was obtained next day and for several days subsequently on which the urine was tested. Thus no trace of sugar could be discovered. Both wounds were healed by June 23rd.

(N.B.—This was the only experiment in which the wounds were not completely healed at the time of parturition).

Leaving out of account Experiment 2, in which the animal was suffering from tubercle and had recently aborted, the experiments indicate that removal of the mammary glands does not bring about a condition of glycosuria after a subsequent parturition, and that at such a time there is normally no increase of glucose in the blood such as one would suppose if Bert's hypothesis were true.

The possibility suggested itself (although it appeared to be exceedingly unlikely) that guinea-pig's milk might be deficient in sugar as compared with goat's milk, since guinea-pigs when newly born are not dependent upon the mother suckling them to nearly the same extent as most mammals, and consequently the milk might be somewhat abnormal. Accordingly we obtained a few drops of guinea-pig's milk by expressing it from the teats, and after diluting it with a little water tested it with Fehling's solution, which gave a very marked reduction. It was not possible to obtain sufficient milk to estimate the amount of sugar, but it was evident that the sugar was present in considerable quantity.

It is known that lactosuria occurs occasionally in women during lactation, and also, according to some, in the later stages of pregnancy (Hofmeister, 1878; Porcher, 1906); and it is usually supposed that the lactose got rid of in this way is derived from the charged mammary

glands, being absorbed into the blood and afterwards excreted by the kidneys. It occurred to us, therefore, that the unidentified sugar found by Bert after parturition in the goat's urine might be lactose which had been found in a small portion of mammary gland that had been left behind accidentally at the time of the operation, and had subsequently undergone hypertrophy.<sup>1</sup> Under such circumstances the milk (if formed) with its contained lactose would have no way of escape, and its constituent parts might become re-absorbed into the blood. However this might be, on isolating a suckling guinea-pig which had recently given birth to young, and on examining its urine, sugar was found to be present in considerable quantity, but the crystals formed on applying the phenylhydrazine test showed that the sugar was for the most part glucose, while lactose, if present at all, was only there in very small amount. On the following day the guinea-pig's urine had become normal, no reduction being given by Fehling's test. On repeating the experiment with a suckling rat shortly after parturition a negative result was obtained, the rat's urine having no reducing action on Fehling's solution.

These experiments show that glycosuria may take place after parturition in normal unoperated animals, but that it does not do so invariably. When glycosuria does so occur, its occurrence is probably comparable to post-operative glycosuria, the cause of which is not understood.

The glycosuria observed by Porcher after removal of the mammary glands during lactation may probably be explained as a post-operative effect, and cannot be cited in support of Bert's hypothesis.

Evidence has been adduced by various authors that the growth of the mammary glands and the formation of milk are the direct or indirect result of a specific stimulus arising in the placenta (Keiffer, 1902; Halban, 1905) or in the foetus (Lane-Clayton and Starling, 1906). In view of the facts cited above it is evident that this stimulus

1. Ribbert (1894) has shown that if in performing the operation of removal of the mammary glands the operator fails to extirpate all the tissue completely an active regenerative process rapidly takes place from the tissue that is left behind.

must act solely on the tissue of the mammary glands, and not upon the liver or some other organ in the body in addition, as one would have to suppose were Bert's hypothesis proved to be correct.

Further, the specific mammary hormone is apparently quite inactive in the complete absence of the mammary glands, and in such cases is ineffective in causing a new formation of gland tissue, for on killing the guinea-pig (referred to in Experiment I) which had become twice pregnant after the extirpation of the glands, not a trace of mammary tissue could be discovered.

#### POSTSCRIPT

Since concluding this paper the guinea-pig referred to in Experiment III became pregnant a second time and had young ones in the middle of November. The young ones were evidently born at full time. The urine of the mother was again tested (for a day after parturition), when no sugar could be detected. Albumen was present in small quantity.

We may note also that Shattock (1905) removed the mammary glands of a guinea-pig which afterwards became pregnant and had young at full time. Shattock carried out his experiment to test the view that the mammary glands elaborate an internal secretion necessary for the growth of the foetus. It will be seen that our experiments confirm Shattock's conclusion that there is no such secretion formed.

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## THE ISLETS OF LANGERHANS IN RELATION TO DIABETES

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(Received October 24th, 1906)

The work recorded in the following paper was undertaken with a view to testing the hypothesis frequently brought forward that the islets of Langerhans of the pancreas yield an internal secretion whose function has some relation to the utilization of dextrose in the vertebrate organism. The primary cause of diabetes is, according to Macleod,<sup>1</sup> the 'want of disruption of the dextrose molecule and its consequent non-utilization by the organism.' Further, it is well established from the experiments of von Mering and Minkowski and others that total extirpation of the pancreas induces violent diabetes in dogs, cats, pigs, and frogs, whilst the supplying of ordinary pancreas *per os* fails to alleviate the condition.

Now, this relation between pancreas and diabetes is held by many to depend more particularly upon the epithelial cell groups, commonly termed 'islets of Langerhans,' which are a characteristic of the pancreas in all vertebrates. These islets have a rich blood supply, and have been held by various writers to be elements *sui generis*. Comparatively recently the view has been advanced (not for the first time by any means) that they are not a distinct tissue, but simply a *phase* of ordinary pancreatic tissue. The present is not an appropriate opportunity upon which to discuss the merits of these opposing views; that is reserved for another occasion. Reference is made to them here simply to clear the ground for what follows. One of us<sup>2</sup> has shown that in Teleostei, at all events, these

1. 'Recent advances in Physiology and Bio-Chemistry,' p. 340, et seq., 1906.

2. Rennie, *Quarterly Journal of Microscopical Science*, 1903.

islets are independent of pancreas proper, and that in several species there exists a particular islet which from its definite position and constancy of occurrence may be termed a 'principal islet.' This islet is in certain fishes of relatively large size, and this, coupled with the fact that in Aberdeen Fish Market we were able to secure exceptional supplies, particularly throughout the winter months, yielded our opportunity. Numbers of *Lophius piscatorius* and other fishes were obtained almost daily, and from these the larger islets were cut out and supplied for consumption to patients suffering from diabetes. They were generally given in the form of a boiled extract; in one case considerable quantities were consumed in the raw state. In each case will be found indicated the particular form in which the islet substance was taken.

Our facts are submitted as a contribution to the problem suggested by the title of this paper. The work was undertaken in view of the known relation between the pancreas and diabetes, and also in view of the frequently assumed relation between these islets when pathologically affected and this disease. While the experiments as they stand cannot be regarded as conclusive in establishing a connection it is well to bear in mind that a decisive answer is scarcely to be looked for from experiments such as these alone. This is so mainly because—

(1) Under the most favourable circumstances we have been able to use *only a very small quantity of islet substance*, e.g., in Case III (period 6), where 4 grams per day were given, but this was maintained only once for a week on end.

(2) There is a possibility that the metabolism of the cold-blooded animals (Teleostei) whose islets were used may differ from that of man to such a degree as to materially affect their action in the cases tested.

(3) With one exception the cases treated were all well established, and of a severe type.

(4) Some difficulty was experienced, as will be seen, in inducing patients to continue under treatment for a sufficient period.

An account has already been published<sup>1</sup> giving the results of experiments testing the action of islet substance *in vitro* upon dextrose, when negative results were obtained.

The sugar estimations in the following cases were made by the Pavy or the yeast methods. In some cases duplicate tests were made by the House Physician at the Infirmary.

*Case I.*—J. H., aet. 18, with a record of this disease for about two years previously, having twice been in Aberdeen Royal Infirmary during that time. Ten months before the commencement of the present treatment he was passing about 400 ounces urine daily, containing about 4,000 grains sugar. This was in the winter of 1901-2. He applied for dispensary treatment and in November, 1902, his urine per 24 hours was 560 ounces, containing 21,500 grains sugar. He had gone almost blind, and was very weak. This was the first case to be treated with these islets and at this time supplies were very limited. Indeed, during the first sixty days of the experiment, the total quantity taken by the patient amounted to only 34.25 grams, or an average of .57 gram daily. The islets were taken from *Hippoglossus vulgaris*, *Anarrhichas lupus*, and *Lophius piscatorius* at first, latterly, entirely from *Lophius*. They were macerated in a mortar and digested for some time at 40° C. The patient was on ordinary diet, except starch, and took the islets three times daily between meals. The following notes indicate the progress of the case:—

1902.

Nov. 13—Patient received .6 gram islets.

„ 19—Urine about 400 ounces. Patient feeling better.

„ 25—Patient in good spirits and his thirst is distinctly diminished. Urine 320 ounces; Sugar 11,187 grains.

„ 26 to Jan. 10—During this period the quantity of urine per day was about 320 ounces, only twice was it as high as 400. The quantity of sugar did not show much fluctuation, an average sample containing 12,288 grains. About the beginning of January it was rather less. The patient's health was somewhat varied and apart from the reduction in the quantity of urine and sugar, and the continued relief from distressing thirst, there were no other features suggestive of improvement.

Jan. 10 to 26—Patient somewhat stronger and showing signs of improvement. Polyuria gradually diminished so that daily quantity is at 260 ounces, and total sugar about 8,240 grains.

„ 27 to 30—Supplies of islets withheld. Urine rose to 320 ounces and thirst was doubtfully increased.

1. Rennie, Zentralblatt f. Physiologie, Bd. xviii., No. 13.



Jan. 30 to Feb. 8—With slight fluctuation the urine fell to 240 ounces and patient appeared improved.

Feb. 9 to 24—During this period no islet substance was supplied. On the whole the general health appeared good, the urine, with the exception of a rise to 280 ounces on the 13th and 14th, slowly fell to 220 ounces. On 19th the sugar amounted to 7,700 grains. Notwithstanding this reduction the patient seemed weaker, and he stated that his blindness was increasing.

„ 25 to Mar. 23—With little fluctuation, the urine fell slowly and steadily during this period. In first week of March his appetite was that of a person in health, and his thirst distinctly reduced. Sugar, on Feb. 26th, was 7,200 grains, on Mar. 4th, 7,000, and on the 19th, about 6,600. On the 23rd the supply of islet substance failed.

Mar. 24 to April 3—No more islet substance obtainable. From the 21st onward he did not feel well, and complained of sickness and failing appetite. On April 1st he went out a short distance, it was a very cold day, and on that evening he complained of severe headache and racking pains in his bones. Ill and in bed till morning of 3rd, when he died.

*Note.*—It will be seen from the introductory note that this was a hopelessly bad case, in which all the known methods of diabetic treatment had effected no result. Little was expected of islet treatment in such a case, but it was the only case available at the time, and the results are as given above.

*Case II.*—Female, aet 45, admitted 28th Jan., 1903, to Royal Infirmary. From 29th Jan. to 12th Feb. patient was under no special treatment. The following particulars regarding her condition during this time were noted :—

Date 1903.	Ounces Urine.	Grains Sugar.	Remarks.
Jan. 29	164	654	Wt. 9 st. 2½ lbs.
30	92	—	
31	182	—	
Feb. 2	120	—	
3	62	1054	
4	83	—	Wt. 8 st. 12 lbs.
8	66	1122	
9	76	—	
10	67	—	
11	—	2848	
12	—	—	Wt. 8st. 12 lbs.

During this period it will be observed that the output of sugar steadily increased, while the patient also lost weight. Supplies of islet substance, prepared as in Case I,

equal to about  $1\frac{1}{2}$  grams per day, were commenced on the 13th, and were prepared and given in the usual way. Annexed are the details recorded for the time the treatment was in use:—

Date 1903.		Ounces Urine.		Grains Sugar.		Remarks.
Feb. 18	...	35	...	700	...	Wt. 9 st. 0 $\frac{1}{2}$ lbs.
19	...	51	...	1071	...	
20	...	68	...	1564	...	
22	...	68	...	1560	...	
23	...	63	...	1827	...	
24	...	53	...	1219	...	
25	...	58	...	870	...	
26	...	49	...	980	...	Wt. 9 st. 0 $\frac{3}{4}$ lbs.
27	...	65	...	1235	...	
28	...	67	...	1139	...	
Mar. 1	...	88	...	1496	...	
2	...	67	...	1330	...	
3	...	40	...	1040	...	
4	...	73	...	876	...	
5	...	54	...	1404	...	
8	...	66	...	924	...	Wt. 9 st. 3 $\frac{1}{4}$ lbs.
9	...	59	...	885	...	
10	...	52	...	780	...	
11	...	59	...	531	...	
12	...	51	...	714	...	Wt. 9 st. 3 $\frac{1}{4}$ lbs.
15	...	46	...	—	...	
16	...	53	...	954	...	
21	...	53	...	530	...	Wt. 9 st. 4 lbs.
22	...	36	...	—	...	
24	...	54	...	702	...	

During this period it will be noted that the sugar is distinctly diminished, while at the same time the weight is increased.

On the 24th our supply of islets was exhausted and no more could at this time be obtained. The patient was kept under observation for some little time longer but could not be induced to remain after the beginning of April. The following are the observations made during this period:—

Date 1903.		Ounces Urine.		Grains Sugar.		Remarks.
Mar. 25	...	66	...	858	...	
26	...	88	...	1144	...	Wt. 9 st. 4 lbs.
27	...	83	...	—	...	
28	...	78	...	—	...	
29	...	64	...	1024	...	
30	...	70	...	—	...	
31	...	79	...	—	...	
Apr. 1	...	69	...	—	...	Wt. 9 st. 4 $\frac{1}{4}$ lbs.

Absence of abnormal thirst was a marked feature after the treatment had commenced.

*Case III.*—G. T., male, age 44. *Diabetes mellitus.* Record previous to commencement of treatment with islet substance is briefly as follows. Four years earlier the patient had business worries and drank to excess for a time, but broke it off. He became listless, easily tired, and drank large quantities of water. His urine was examined for the first time in July, 1901, and found to contain large quantities of sugar (4,800 grains in 24 hours). For a time he went on strict diet and the output of sugar ceased. It appeared occasionally afterwards and later became permanent. The patient tired of strict dieting and ate sugary and starchy foods. His weight remained fairly constant (about 11 stone) for the two years previous to the commencement of the experimental treatment in May, 1904. In the early part of that year he had an attack of congestion of the liver. At the beginning of treatment he was able to walk nearly two miles slowly, without feeling too fatigued. For a considerable period of time he was supplied with about  $1\frac{1}{2}$  grams islets daily. The islets at first were boiled, acidified with weak acetic acid and filtered. The filtrate was taken three times daily, between meals. Subsequently, as will be seen, the islets were taken in the raw state. Urine and sugar estimations were made as frequently as possible, the full details of which are given below. In order to render the study of this record easier it is given in periods, each of which corresponds to some modification of the treatment.

(1). *Daily supply of  $1\frac{1}{2}$  grams (gross wt.) boiled and proteid free islets.*

	Date 1904.		Ounces Urine.		Grains Sugar.		Remarks.
May	3	...	225	...	6750	...	Temp. 97·8; Pulse, 90.
	4	...	210	...	4095	...	Temp., 98·4; Pulse, 86.
	5	...	204	...	4692	...	
	6	...	280	...	4900	...	Weight, 10 st. 9 lbs.
	7	...	250	...	5250	...	
	9	...	240	...	5520	...	
	11	...	225	...	6862	...	
	14	...	225	...	6525	...	
	15	...	220	...	6820	...	
	17	...	200	...	5200	...	Wt. 10 stone. 11 lbs.
	18	...	190	...	3610	...	
	20	...	205	...	2255	...	
	21	...	190	...	3325	...	
	25	...	195	...	3413	...	Wt. as before.
	28	...	190	...	2850	...	
June	1	...	195	...	3705	...	
	5	...	190	...	3325	...	Wt. as before.
	8	...	180	...	3510	...	
	12	...	190	...	2470	...	Wt. 10 st. $9\frac{1}{2}$ lbs.
	16	...	195	...	3413	...	
	20	...	190	...	3230	...	Wt. 10 st. 9 lbs.
	27	...	200	...	3500	...	



Date 1904.		Ounces Urine.		Grains Sugar.		Remarks.
July	20	...	170	...	5950	...
	23	...	140	...	3675	...
	26	...	165	...	3135	...
	29	...	145	...	2900	...

The quantity of islets given was very small during this period. The sugar output shows a distinct falling off, so also the quantity of urine passed.

(2.) *Period of Five weeks during which no islet substance was taken.* (Sept. 1 to Oct. 8).

Date 1904.		Ounces Urine.		Grains Sugar.		Remarks.
Sept.	1	...	150	...	2850	...
	8	...	160	...	4200	...
	18	...	165	...	5445	...
	25	...	155	...	5735	...
Oct.	3	...	—	...	—	...
	8	...	115	...	2185	...

Probably exhaustion the factor here in the diminution of sugar, as this is frequently noted in diabetic cases.

(3.) *Filtered extract of islet substance in Normal Saline, given hypodermically.*

Date 1904.		Ounces Urine.		Grains Sugar.		Remarks.
Oct.	9	...	110	...	2090	...
	10	...	120	...	3150	...
	11	...	—	...	3150	...
	12	...	115	...	3020	...
	13	...	—	...	—	...
	14	...	120	...	2520	...
	15	...	115	...	3450	...
	16	...	115	...	3020	...

This was purely experimental. The amount of reaction caused by the hypodermic injection, accompanied as it was with no improvement such as we appeared to get by other methods of administration, caused us to abandon this mode of treatment.

(4.) *Daily supply of  $1\frac{1}{2}$  grams boiled unfiltered islet substance, in three doses, two hours after food.*

Date 1904		Ounces Urine.		Grains Sugar.		Remarks.
Oct. 17	...	160	...	2090	...	Pulse 90.
19	...	120	...	2040	...	Pulse 80.
21	...	115	...	1955	...	
23	...	115	...	2415	...	Pulse 88.
25	...	125	...	1875	...	Wt. 10 st. 11 lbs. Stomach and bowels satisfactory. Thirst is never now a distressing symptom; patient feels well.
28	...	130	...	2730	...	
30	...	125	...	2125	...	
Nov. 1	...	120	...	2040	...	
3	...	125	...	2625	...	
6	...	120	...	2400	...	Sleeping and eating well; stronger, no depression.
8	...	130	...	2730	...	
10	...	125	...	2500	...	Wt. 10 st. 11 lbs.
12	...	130	...	2340	...	Note on diet.—Patient taking one slice white bread to tea, one large or two small potatoes at dinner, and occasionally oatmeal porridge for supper.
14	...	135	...	2295	...	
16	...	140	...	2660	...	
19	...	130	...	2470	...	
21	...	135	...	2565	...	
23	...	140	...	2660	...	
25	...	125	...	2125	...	
Dec. 2	...	135	...	2565	...	
8	...	115	...	1955	...	Extremely well; standing cold weather well.
11	...	130	...	2470	...	
13	...	—	...	—	...	Supply of islets stopped

In spite of the additional carbohydrate in diet the diminished quantity of sugar is maintained during this period.

(5.) *Brief period without supplies of islets.*

Date 1904.		Ounces Urine.		Grains Sugar.		Remarks.
Dec. 15	...	135	...	2835	...	No islets since 13th.
18	...	140	...	3220	...	
20	...	—	...	—	...	General health good. It is note- worthy that both the tests for sugar this week show a higher figure than was reached during the whole of the previous month, as is shown above.

(6.) *Period during which patient received islets raw, macerated in Normal Saline. It was found impossible to maintain a daily supply, although a much larger quantity was consumed than at any previous time. Unless otherwise stated below, the day's supply when given was about 4 grams fresh islets.*

Date 1904.	Ounces Urine.	Grains Sugar.	Remarks.
Dec. 23	...	...	4 grams taken.
24	135	2160	None.
25	135	3105	None.
26	130	2470	None.
27	120	3120	4 grams taken.
28	120	2160	4 grams taken.
29	120	2280	4 grams taken.
30	115	1840	4 grams taken.
31	125	2125	4 grams taken.
Jan. 1	130	2080	4 grams taken.
2	130	2340	4 grams taken.
3	125	1875	None taken.
4	125	2000	None taken.
5	120	2520	4 grams taken.
6	130	2600	4 grams taken.
7	120	2280	4 grams taken; Wt. 10 st. 10 lbs.
8	130	2210	4 grams taken; Patient out every good day, feels strong, no weakness in legs.
9	135	2295	None taken.
10	120	1680	None taken.
11	135	2295	1½ grams taken.
12	115	2300	4 grams taken.
13	130	1950	4 grams taken.
14	120	1920	4 grams taken.
15	130	1950	4 grams taken.
17	125	2250	4 grams taken.
20	130	2600	5½ grams in 3 days.
23	135	2700	2½ grams on 21st, 1½ grams on 23rd.
26	130	1430	4 grams on 24th, 2½ grams on 25th. 1½ grams on 26th.
28	120	1680	2½ grams on 27th, none on 28th.
31	125	2125	None on 29th and 30th, 4 grams on 31st.
Feb. 2	115	1955	4 grams on 1st, 1½ grams on 2nd.
4	120	2160	4 grams on 4th, 4 grams on 3rd.
7	125	1625	8 grams between 5th and 7th.
9	115	1955	8th and 9th, 4 grams in all.
11	115	1495	4 grams on 10th, 2½ grams on 11th; Wt. 10 st. 10½ lbs.
14	120	2040	None on 12th, 4 grams on 13th, 1 gram on 14th.



Date 1904.		Gunces Urine.		Grains Sugar.		Remarks.
Feb. 16	...	130	...	2210	...	15th and 16th, 8 grams in all.
18	...	125	...	2125	...	17th and 18th, 8 grams in all.
21	...	115	...	1783	...	3 grams on 19th.
23	...	130	...	2080	...	None on 20th, 21st and 22nd; 1½ grams on 23rd.
25	...	140	...	1820	...	4 grams each on 24th and 25th.
28	...	135	...	2228	...	26th, 27th and 28th, 4 grams each day.
Mar. 2	...	125	...	2125	...	None; 2½ grams on 1st.
4	...	135	...	2295	...	4 grams on 3rd, 4 grams on 4th.

During all this period the diminution in sugar is well maintained, as well as weight, but it was soon apparent that we had come to the limit of reduction so far as this case was concerned.

(7.) *Period of nine days without supplies of islets.*

Date 1904.		Ounces Urine.		Grains Sugar.		Remarks.
Mar. 7	...	135	...	2295	...	Last supply on 4th.
9	...	145	...	2248	...	
11	...	145	...	2610	...	
13	...	—	...	—	...	No test; on 14th, sugar 3150 grains.

There was no apparent recovery from this point.

(8.) *Same as period 6, but towards the end the supplies were considerably reduced.*

Date 1904.		Ounces Urine.		Grains Sugar.		Remarks.
Mar. 14	...	150	...	3150	...	4 grams taken each day from 14th to 18th.
16	...	150	...	2850	...	
18	...	135	...	2835	...	
21	...	145	...	3045	...	None taken for the five days, 19th to 23rd.
23	...	150	...	2550	...	
25	...	145	...	2610	...	For nine days, 24th to April 1st, the average supply per day was 2 grams.
27	...	135	...	2565	...	
30	...	140	...	2660	...	
April 1	...	130	...	2600	...	
3	...	145	...	2610	...	4 grams on 2nd.
6	...	120	...	2280	...	None on 3rd, 4th, 5th, 4 grams on 6th, 4 grams on 7th, after which no further supplies were given. Wt. on 4th, 10 st. 7 lbs.
8	...	120	...	2040	...	

*Further Notes.*—About the 23rd March the patient felt only fairly well, and was languid. He does not appear to have, at any subsequent time, been quite so well as usual, and on the 10th April he was sick and vomiting during the night, with stomach pains. On the morning of the 11th he was better, but again took ill during the night. On the morning of the 12th coma, and death at 6-30 p.m.

Case II.—J. L., age 50—*Diabetes mellitus*.—The following statement indicates the progress of the patient for the period during which he was under observation and treatment :—

Urine per 24 hours. (7 days' average)		Grains sugar per 24 hours. (7 days' average.)		Remarks.
177	...	2675	...	Diet ordy., no carbohydrate. Temp. 97.2 to 98.3, lost 5½ lbs. in wt.
141	...	3513	...	20 m. liq. morph. t.d., lost 1 lb. in wt.
124	...	3987	...	20 m. liq. morph. every 6 hrs., gained 2 lbs. in wt.
151	...	4574	...	Morph. every 6 hrs., lost 1 lb. in wt.
137	...	3612	...	40 m. liq. morph., 2 drachms cod liver oil nightly, lost 5 lbs. in wt.

For the seven days prior to the commencement of the treatment with islet substance the figures are :—

Ounces Urine.	Average.	Grains Sugar.	Average.	Remarks.
145	135	3480	3516	Diet as before. No Drugs ; lost 4 lbs.
156		3432		
170		3910		
110		1980		
156		5304		
114	96	3420		
96		2784		

The following are the corresponding figures for the period during which the patient remained under treatment *with islet substance* ; boiled extract was given, but only small quantities were available at the time.

Ounces Urine.	Average.	Grains Sugar.	Average.	Remarks.
134	126	3484	3367	No drugs and no alteration in diet ; gained ½ lb.
148		3848		
165		4125		
119		2975		
80		2240		
104	99	3120	2765	
134		3752		
140		4060		
94		2444		
64		1792		

Patient, at this stage, left the hospital, declining to remain longer under treatment.

This case shows the effect of the usually adopted method of treatment ; it may be noted that very great fluctuation in the amount of sugar, even on successive days, are common features of these cases, *e.g.*, one day 1980 grains and the next 5304 grains. While the patient is taking islets these fluctuations are much less marked, and a general trend in a downward direction was noted so long as the patient could be induced to stay in hospital.

*Case V.*—W. M., female, age 59—*Glycosuria*.—The following is the record of this case while the patient was under treatment:—

(a) *Prior to treatment with islet substance.*

1904.	Ounces Urine.	Average.	Grains Sugar.	Average.	Remarks.
Jan. 30	...	60	960	743	During this period the patient lost $3\frac{1}{4}$ lbs.; Diet: antidiabetic.
31	...	66	990		
Feb. 1	...	80	1120		
2	...	60	920		
3	...	57	684		
4	...	54	432	402	Temp.: nothing specially noteworthy; Pulse: 68 to 96.
5	...	74	296		
6	...	51	306		
7	...	68	476		
8	...	50	400		
9	...	40	240	55	
10	...	54	432		
11	...	80	640		
12	...	40	320		

(b) *Period of treatment with islets.*

13	...	55	440	127	At end of this period, patient had gained $\frac{1}{4}$ lbs.
14	...	52	312		
15	...	46	46		
16	...	52	52		
17	...	40	40		
18	...	52	—	None	Temp.: as before; Pulse: much steadier, 68 to 82; Diet ordinary during this period.
19	...	48	—		
20	...	45	—		
21	...	49	—	None	
22	...	46	—		
23	...	47	—		
24	...	52	—		
25	...	50	—		
26	...	50	—		

Six weeks later patient reported 'well.'

This case had none of the symptoms usually associated with *Diabetes mellitus*, save the glycosuria. Special diet was evidently proving satisfactory up to the time the islets were started, though there was a distinct loss of weight. The disappearance of the sugar is more markedly rapid after islet treatment came into effect.



In concluding this series we take the opportunity of stating that the cases were not entirely under our care, but that permission to give the treatment and make observations was kindly given by Professor Finlay and Dr. Edmond, Physicians at the Aberdeen Royal Infirmary ; Dr. W. R. Pirie, Aberdeen Dispensary ; and Dr. Lilius Thomson, Edinburgh ; and for whose courtesy we desire to express our thanks. We also beg to acknowledge with thanks grants from the Carnegie Trustees, with which our expenses were defrayed.

## NOTE ON THE OCCURRENCE AND CONSTITUTION OF LIPÖID MATERIAL IN DIABETIC BLOOD

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(Received October 29th, 1906)

The condition known as Lipaemia, occurring in Diabetes, has hitherto not been regarded as a common one, and its presence has usually been regarded as of serious significance. During the course of some observations on the alkalinity of the blood, one of us (F.P.W.) had occasion to take samples from six cases of Diabetes Mellitus. This blood, and that in the additional cases, was collected in Wright's capsules.

Almost immediately after withdrawal from the patient the blood in five of the cases appeared opalescent, and in one there separated out a white purulent-looking material, which from mixture with red corpuscles presented an appearance like crushed strawberries and cream.

As most of these cases had been improving under treatment, and showed no signs of coma, we determined to investigate the subject further.

Accordingly, we have examined the blood of fifteen cases of Diabetes, and in ten the above condition was present in varying degree.

One of the series, Case XII, had only a temporary glycosuria.

Most of the cases showed only a semi-translucent opalescent appearance. This is easily distinguished, once it has been observed, from the fat-laden serum which separates out from normal blood after a meal of fat. The difference is still more marked after centrifugalisation; the former retaining its opalescent character, the latter separating to yield clear serum.

The condition seemed to vary in degree from time to time, and in three cases disappeared entirely. Two cases in which it was absent at first showed it later.

The patients were all adults except Case IX, a boy aged twelve years.

Cases I to XII were in the Royal Infirmary, and Cases XIII to XV in Mill Road Infirmary, Liverpool.

The following Table shows the conditions under which the presence or absence of the lipaemia occurred:—

Number of Case	Initials of Patient.	Date.	Diet.	Presence of Lipaemia.	Sugar per diem.	Acetone.	Di-acetic Acid.	Treatment.	Fat in Diet.
I	P. McC.	April 3	Diabetic	+	5,700	+	+	Duodenal Extract Phenazone	+
II	J. O.	April 3	Diabetic	+	2,800	+	+	Duodenal Extract Antipyrin	+
III	E. B.	April 3	Diabetic	+	8,346	+	+	Nil	+
IV	L. W.	May 10	Diabetic	+	8,545	+	++	Duodenal Extract Antipyrin	+
V	S. S.	May 10	Ordinary	—	2,612	+	+	Nil	
VI	G. C.	July 6	Ordinary	+	4,219	+	+	Nil	
VII	W. M.	April 3	Diabetic	+	—	+	+	Alkalies	+
		May 8	Diabetic	+	7,788	+	+	Duodenal Extract	
		July 4	Diabetic and Potatoes	+	3,967	+	+	Duodenal Extract	
VIII	W. Y.	May 10	Diabetic	+	8,194	+	+		
		July 12	Diabetic	+	3,534	+	+	Duodenal Extract Alkalies	+
IX	T. M.	April 3	Diabetic	—	3,420	—	—	Nil	+
		May 8	Diabetic	+	4,000	+	+	Duodenal Extract— HCl	+
		July 7	Diabetic	+	4,864	+	+	Duodenal Extract	+
X	J. F.	May 22	Ordinary	+	6,714	+	+	Nil	
		July 7	Diabetic	—	not estimated	—	slight	Duodenal Extract— HCl	+
XI	M. P.	April 3	Diabetic	+	1,341	+	+	Duodenal Extract Phenazone	+
		July 12	Diabetic	—		+	+		+
XII	A. B.	May 8	Diabetic	—	Present	—	—	Nil	little
		May 22	Diabetic	—		—	—	Duodenal Extract	little
XIII	"B," 2	April 8	—	—	Present	—	—		little
XIV	"B," 3	April 8	—	—	Present	—	—		little
XV	"D," 3	July 2	—	—	Present	—	—		little
		July 7	—	—		—	—		much

## NOTES TO THE TABLE

*Relationship to Diet.*—Most of the cases we examined had been on diabetic diet for some time, but three had the condition on admission to the Hospital. Some cases had the fat in their diet considerably reduced, but it made no apparent difference to the blood. Cases XIII and XIV were not lipaemic when their blood was examined, and, unfortunately, died two or three days later. Case XV, also not lipaemic, was allowed a fat-rich-diet, but five days later the lipaemic condition was absent, and has remained so. It is obvious that *drugs* did not produce the condition, as some of the patients had received no drugs at all. There is no apparent relation between the condition and the amount of sugar excreted.

*Acetone and Di-acetic Acid.*—The Table shows a very striking relation between the lipaemic condition and the presence of acetone and di-acetic acid in the urine. Cases V and XI (on one occasion), which are exceptions, were extremely ill when the observations were made, and it is interesting to note that in the severest cases the blood condition was absent. Case IX is instructive in that it showed at first the absence of acetone and di-acetic acid, and also of lipaemia, while later these conditions occurred concurrently. For this reason we decided to inquire into the effect of fat in the food on the lipaemia and on the substances in the urine. Case XII was kept on a fat-rich-diet for eleven days, and the amount of acetone estimated. Then fat was removed as completely as possible from the diet for six days, and the effect noted. Unfortunately, during the latter part of the investigation the patient surreptitiously obtained some potatoes, but, so far as we could ascertain, no fat.

The following amounts of acetone were excreted per diem :—

1st day ... ..	1'048	grms.	6th day ... ..	7008	grms.
2nd „ ... ..	1'57	„	7th „ ... ..	9916	„
3rd „ ... ..	957	„	8th „ ... ..	8254	„
4th „ ... ..	1'061	„	9th „ ... ..	9035	„
5th „ ... ..	1'032	„	10th „ ... ..	796	„
11th day ... ..			796		

On the Fat-free Diet :—

12th day ... ..	1'4054	grms.	15th day ... ..	1'417	grms.
13th „ ... ..	1'1708	„	16th „ ... ..	1'298	„
14th „ ... ..	1'188	„	17th „ ... ..	1'234	„

It is thus evident that the acetone did not diminish with the cutting off of the fat. The blood condition also was unaltered.

A second observation to the same effect made on Case VI, under more stringent conditions, yielded similar results.



## THE NATURE OF THE LIPÖID MATERIAL

*Microscopical Examination.*—The material was found to consist of minute globules, smaller than those of milk, in a state of movement resembling the Brownian movement.

*Chemical Examination.*—The blood used for this purpose was from Case X. It was one of the less marked cases, merely showing the opalescent appearance noted above. Sir James Barr, under whose care the patient was, kindly obtained for us 28·649 grams of blood. This was extracted with alcohol and ether, the weight of the total extract when dried being 1 per cent. of the whole blood.

An analysis of this extract showed it to contain—

18·9 per cent. of free fatty acid,

60·2 per cent. of combined fatty acid,

Both estimated as oleic acid.

The unsaponifiable matter having been separated, pure crystalline cholesterin was isolated from it, the amount being 18·1 per cent. of the ethereal extract.

In two of the most marked cases cholesterin was isolated from about 1 c.c. of the blood, and recognised microscopically. The fatty acids gave an iodine value of 27, which shows that the bulk of the fatty acids present were saturated. Dr. H. E. Roaf, in a case of Lipaemia from the post-mortem room, recently found an uncrystallisable material giving the reactions of cholesterin, and which was probably one of its isomers.

B. Fischer<sup>1</sup> in one case of Lipaemia with coma found the blood to be of the same nature, the lipoid material being mainly composed of an ester of cholesterin, and also that the fatty acids present were saturated.

It is usually accepted that the probabilities are that saturated fatty acids come from the body-fat, whereas the unsaturated fatty-acids are more likely derived from the food-fat.

It is, therefore, probable that the fatty-acids in lipaemia are drawn from the tissue fats.

<sup>1</sup> *Virchow's Archiv*, Bd. 172, pp. 30-71, 218-261.

Our observations tend to confirm this, as in the severe cases, where most of the body fat had been used up, the blood did not show the condition of lipaemia.

In conclusion we should like to draw attention to the following deductions :—

1. That the occurrence of the so-called lipaemia is not so rare a condition, nor of such grave significance, as has formerly been supposed.
2. That it is in all probability an expression of an abnormal metabolism of fat; as the character of the ester in the blood and its presence concurrently with acetone and di-acetic acid in the urine would show. This would explain the danger which is said to occur from sudden withdrawal of all carbohydrates from a diabetic diet, for the patient has then to draw upon his tissue fat, and in so doing would accentuate this abnormal metabolism.

We have to thank Sir James Barr, Dr. Bradshaw, Dr. Abram, and Dr. Raw for the use of their cases, and also Professor Benjamin Moore for his kindness and advice in his laboratory, where the work was carried out under his direction.

## THE EFFECT OF ARTERIAL OR VENOUS OBSTRUCTION UPON THE NUTRITION OF THE LIVER CELLS

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### INTRODUCTION

These experiments were undertaken in order to ascertain more definitely the relative parts played by the arterial and venous blood, which supply the liver, in the nutrition of the liver cells ; and attention was directed more especially to the appearance of fatty changes in these cells. Widely differing views have been held by various writers as to the extent to which the hepatic artery directly supplies arterial blood to the liver cells. The observations of Cohnheim and Litten led them to believe that no arterial blood entered directly the capillary network of the lobules ; they supposed that the hepatic artery supplied blood to the connective tissue framework of the liver, and nourished the walls of the portal vessels running in that framework. Rindfleisch, however, concluded—on the ground of the changes occurring in amyloid disease—that the hepatic arterioles joined the capillary network in the middle zone of the lobule, and that the outer zone received only portal blood.

Recently, Kowalewsky and Opie have independently come to the conclusion that both the artery and the portal vein supply the entire lobule.

Cohnheim and Litten, working with rabbits, found that ligature of the hepatic artery caused necrosis of the entire liver, and death in twenty-four hours. After tying the artery going to a single lobe, the animals lived two or three days. The lobe was necrotic, and showed scattered haemorrhages ; no fatty change was observed.

They concluded that after cutting off the arterial blood supply the nutrition of the walls of the portal veins was impaired. The blood, therefore, stagnated and clotted in these veins, so that anaemic necrosis of the liver occurred.

Ligature of a branch of the portal vein caused death in 24 hours. But the production of multiple embolism in the liver by the injection of wax into the portal vein left the liver cells unaffected. On the other hand, Solowieff, after ligature of a branch of the portal vein, observed at first atrophy of liver cells, and later general fibrosis of the interlobular connective tissue.

More recently, Doyon and Dufourt observed that ligature of the hepatic artery in dogs caused death in twenty-four to forty-eight hours. In such cases areas of necrosis were observed. The results were not constant owing to variability in the collateral anastomoses. In successful cases the proportion of urea in the urine to the total output of nitrogen was diminished. Ligature of a branch of the portal vein caused no necrosis.

Tischner found that in rabbits ligature of the hepatic artery caused thrombosis of branches of the portal vein owing to stagnation of the blood, and necrosis occurred. Infarction occurred in some of the lobules, and the liver cells round the infarcted area showed an increase of fat.

In dogs, Dujarier and Castaigne found that ligature of the hepatic artery, under aseptic conditions, had no effect, but that if sepsis supervened necrosis occurred.

In cats, Ehrhardt obtained general necrosis of the liver after tying the hepatic artery.

Ligature of a branch of the portal vein caused decrease of the fat in the liver, and damaged the nutrition of the cells. After two to three months cirrhosis occurred, and even ascites.

There is no doubt that the collateral blood supply to the liver, after tying the main hepatic artery, varies greatly in different species of animals, and even in different animals of the same species; it is least developed in the rabbit, and most so in the dog. These variations largely account for the divergent results obtained by various



observers. Most investigators have found, however, that the occlusion of arterial blood is followed by acute necrosis of the liver, whereas ligature of a branch of the portal leads after a considerable time to atrophy of the liver cells and to fibrosis.

## METHODS

*A. Experimental.*—Most of the experiments were carried out on cats; three dogs were employed. The cats were anaesthetised with ether only, and the dogs with A.C.F. mixture after a preliminary dose of  $\frac{1}{2}$ -1 gr. morphia, the anaesthesia being subsequently maintained with the same anaesthetic. Strict antiseptic precautions were taken throughout the experiments, and healing always occurred by first intention, except in the case of one dog, which became infected and was killed on the day after the operation.

The hepatic artery was tied one-quarter to half an inch beyond its origin from the coeliac axis as it passed forward towards the gastro-hepatic omentum. In some experiments, which will be referred to later, all the visible small arteries running to the liver in the gastro-hepatic omentum were also ligatured.

In two experiments only that branch of the artery was tied which supplies the right lobe of the liver.

In many experiments a small piece of the liver was snipped off for microscopical examination at the time of the operation, and served as a control in studying the histological changes resulting from the ligature. In two experiments most of the left lobe of the liver was removed at the time of the operation.

*Portal Vein.*—The right lobe of the liver receives a large branch of the portal vein, which can readily be isolated and tied without disturbing the arterial supply to that lobe or interfering with either the other vascular connexions of the liver or the bile ducts.

The effects of ligature of this branch were confined to the right lobe, so that the rest of the liver served as a control.

Portions of the liver removed at the time of operation and after death were hardened in Müller's fluid; frozen sections were cut and

stained with Soudan III; other pieces were treated by Marchi's method, or stained with the ordinary dyes.

*B. Chemical.*—The pieces of liver taken for the fat determinations were freed from blood, dried, and powdered, and the powder dried in vacuo at 100° C. The dry powder was weighed and extracted with chloroform in a Soxhlet apparatus after being boiled for a short time in alcohol, according to the method recommended by Rosenfeld. The extract so obtained was then boiled with alcoholic potash, and the fatty acids insoluble in water, set free by acidification of a dilute aqueous solution of the soaps, were collected, taken up in light petroleum, freed from solvent, and weighed. The weight of the insoluble higher fatty acids thus obtained was calculated in percentage of the total solids of the tissue.

## RESULTS

### *A. Ligature of the Hepatic Artery*

Owing to the presence of small collateral arteries supplying the liver in cats it is difficult, perhaps impossible, absolutely to shut off all arterial blood from the liver. Consequently, the morbid changes observed vary in degree corresponding to the extent to which arterial blood is excluded. Ligature both of the main artery and of all visible collaterals causes death in twenty-four hours, and very intense changes in the liver cells; ligature simply of the main artery modifies the nutrition of the liver cells to a variable extent in different animals. The pathological alterations vary greatly in degree, and in any case last only for a few weeks, since recovery takes place by the development of the collateral circulation.

The morbid appearances to be described are the result, therefore, of a partial (and variable) deprivation of the arterial blood supply.

*I. Histological Changes.*—The two points observed which seem of most importance are in the first place the increase in the amount of fat visible in stained sections, and secondly the absence of gross necrosis of the liver. When death occurred within twenty-four hours the liver looked abnormally pale to the naked eye; no haemorrhages or necrotic areas were visible.

Microscopically the portal vessels supplying the lobules were very full of blood ; some, but not all, of them were thrombosed. The columnar arrangement of the cells had to some extent disappeared. The cell-nuclei stained well throughout the lobule, except in the inner zone, where some necrotic cells were visible, which stained very badly. There was, however, no necrosis of the lobule as a whole, although all the cells were obviously severely damaged. In the outer zone of the lobules the protoplasm was shrunken, granular, and apparently free from glycogen ; in the inner zone many of the cells contained large vacuoles ; sections stained with Soudan III showed abundance of fat in the inner zone, corresponding to the vacuoles observed in paraffin sections.

In one experiment, in which the occlusion of arterial blood was almost complete, there was no increase of fat histologically ; even in this case there was no general necrosis.

If the animals survived more than twenty-four hours they were killed by chloroform from two to twelve days after the operation. In such experiments the liver looked pale and fatty on inspection.

Microscopically, the outer and middle zones of the lobules were normal in appearance ; the inner zone contained abundance of fat. A few days after the operation the fat existed chiefly in the form of large globules ; later on it was found as small drops only, and in gradually diminishing amount. In animals killed two or three days after the operation scattered necrotic cells were visible in the inner zone close to the intralobular vein ; some of the cells also contained granules of brown pigment, probably bile.

With one exception, the capillary network was not unusually engorged with blood, and the general appearance of the liver was not that of infarction. In one experiment the animal was killed twenty-six days after the operation. The liver appeared normal both microscopically and on section. This may have been due to recovery by the establishment of a collateral circulation, or the animal may, in the first instance, have possessed a good collateral blood supply, so that ligation of the main hepatic artery only slightly damaged the liver.

It should be pointed out that in normal cats the amount of fat found (histologically) in the liver is extremely variable; in some livers the entire lobule may be full of drops of fat, in others the fat may be almost confined to the outer or inner zone, and in others hardly any fat may be visible. It is most desirable, therefore, to snip off a small piece of liver as a control at the time of the operation; even this, however, is not satisfactory if the control piece is full of fat.

Metabolic changes have been described in animals after ligation of the hepatic artery. In our experiments the urine contained traces of sugar for a day or two after the operation; later the urine was normal, and the relation of urea to the total nitrogen was unaltered. For a few days the animals lost weight; subsequently, however, they regained or even exceeded their original weight.

### *B. Ligation of the Portal Vein*

The lobe which was deprived of its portal blood never showed gross necrosis, nor did any appreciable increase in the amount of fat take place.

The animals usually survived, but one or two died within 24 hours. In such cases the columnar arrangement of the cells was lost; the cells throughout the lobule stained badly, and many of them were necrosed, and the lobules as a whole looked atrophied, as though many cells had broken down altogether and disappeared. These necrotic cells were scattered throughout the lobule, and were not confined to any one zone. A few globules of fat were visible, but they were not numerous.

Four days after the ligation the outer zone appeared normal; in the middle and inner zones the nuclei stained well, but the cell substance stained very lightly indeed. Sections stained with Soudan III showed practically no increase of fat. Eighteen days after the ligation the liver was tough and firm, the portal canals were very obvious, and the lobe appeared atrophied. Microscopically, the liver showed a general fibrosis round the arteries and ducts of the portal canals, and the interlobular connective tissue was everywhere increased in amount.



The inner zone was represented chiefly by extravasated blood; the liver cells were scanty, stained badly, and often contained pigment granules. There was no increase in the amount of fat.

The analytical determination of the amount of fat was carried out by the method described above in altogether four animals in which the hepatic artery was tied, and in one in which the vessel ligatured was the branch of the portal vein supplying the right lobe.

In one only of these experiments was the absolute amount of fat in the liver found to be increased after the ligature.

*Experiment No. 4* [January 24].—The branch of the hepatic artery to the right lobe was tied, and two days later the fat estimated in this lobe and in two portions of the rest of the liver. In the former the insoluble higher fatty acids amounted to 21·3 per cent. of the dry substance, in the latter to 14·8 and 14·5 per cent. respectively.

But since the characteristic microscopic appearance of fatty change was not evident in this case, the increase in the fat here does not help us to determine the nature of this change.

In all the other cases the amount of fat was either unaltered or diminished, and it was diminished in that case in which the histological appearance of increase was most marked.

*Experiment No. 1* [October 28].—The left lobe, removed at the time of the operation in which the hepatic artery and its branches were tied, contained 24·6 per cent. of fat; two days later two samples of the liver contained 19·15 and 19·5 per cent. respectively.

*Experiment No. 3* [December 8].—The branch of the hepatic artery supplying the left lobe was tied; two days later this lobe contained 10·5 per cent., and the rest of the liver 10·4 and 10·25 per cent. in two portions taken for analysis.

*Experiment No. 5* [July 3].—The left lobe, removed when the hepatic artery and all its branches were ligatured, contained higher fatty acids, amounting to 11·2 per cent. of the dried liver substance. The liver at death on the next day contained 9·7 per cent.

*Experiment No. 2* [December 4].—The branch of the portal vein to one lobe was tied. Two days later this lobe contained 13·7 per cent., and the portions of the rest of the liver analysed 13·0 and 13·6 per cent. respectively.

## CONCLUSIONS

(1) Ligature of a branch of the portal vein is followed by atrophy of the liver cells, and by fibrosis of the interlobular connective tissue, probably as a replacement fibrosis. It is possible, therefore, that in some cases of hepatic cirrhosis associated with portal thrombosis the thrombosis precedes rather than follows the cirrhosis.

(2) Ligature of the hepatic artery produces (a) necrosis of scattered cells in the inner zone, but no general necrosis of the liver, even when the animals die within twenty-four hours, and (b) considerable injury to the whole of the inner zone, as indicated by the increase of fat (histologically) in the cells.

As regards the necrosis, it is probable that complete occlusion of all arterial blood would cause general necrosis of the liver ; in our experiments this condition was never absolutely fulfilled, and the bulk of the liver cells, though severely damaged, were not killed. There is no doubt that a partial occlusion of arterial blood, if associated with septic infection, can set up necrosis of the liver ; and the combination of these two factors may account for the results obtained by some observers, as was pointed out by Dujarier and Castaigne.

The possible sources of the apparent excess of fat are (1) an infiltration from without ; (2) the formation of fat from proteid by the process of ' fatty degeneration ' hypothesised by Virchow ; and (3) the setting free in a form recognisable by the microscope of fat previously bound up in some complex combination in which it is not detectable by microscopic examination—a process comparable with the liberation of simple fats from myelin in nerve degeneration.

The chemical analyses show clearly that there is no *real* increase of fat in the liver ; the fat, therefore, cannot be either an infiltration or a product of ' fatty degeneration ' of proteid. We consider that when the liver cells are deprived of arterial blood a process of autolysis occurs whereby pre-existent fat recognisable by chemical analysis is set free in a form which stains with Soudan III. It seems clear that this autolysis results from the lack of oxygen, since it is not produced by portal obstruction ; and as the deficiency of oxygen is most marked in the centre of the lobule, the inner zone is chiefly affected.

A very similar condition of the liver (histologically) has been observed by Drummond in animals, and by Langdon Brown in man after intravenous injections of adrenalin ; it may be surmised that in this case, too, the effect is partly the result of a deficient arterial blood supply to the liver.

There was nothing to suggest that the fatty changes were due to toxic or infective causes, since the animals rapidly recovered after the operation, and showed no signs of sepsis.

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## DIRECT MEASUREMENTS OF THE OSMOTIC PRESSURE OF SOLUTIONS OF CERTAIN COLLOIDS<sup>1</sup>

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The statement is often to be found in recent contributions to the chemistry of colloidal solutions, that the colloidal solution is not a 'true solution,' but merely a suspension of colloidal particles in the solvent, possessing no osmotic properties such as are shown by true solutions of crystalloids.

This statement indicates that a hard and fast line can be drawn between colloids and crystalloids, and that the difference is not merely one of degree, arising from the great size of the suspension particle in the case of the colloid, as contrasted with the small size in the case of the molecule of the crystalloid.

There exists, however, at the present time not a particle of experimental evidence for the view that colloidal solutions are *qualitatively* different from crystalloidal solutions. Evidences of quantitative differences exist in abundance; between a typically colloidal and a typically crystalloidal solution the differences are many and striking, but between the two types there are many transitions without an obvious break anywhere, and as the character of the dissolved substance varies so do the osmotic and all the other physical characters of solution vary without a sudden change being detectable at any point.

To put an extreme case; there is no evidence that a visible suspended particle in an emulsion does not exert the same osmotic

1. The expenses of this research have been partially defrayed from a grant allotted to one of us by the Government Grant Committee, Royal Society.



effect as a molecule of a dissolved crystalloid, that the usual indirect methods do not show any osmotic pressure in an emulsion may simply depend upon the fact that the pressure on account of the low concentration of the emulsion particles is too small to be determined by such methods.

It will be shown in this paper, that when the more delicate method of direct measurement of osmotic pressure is used solutions of many colloids show a very distinct and definite pressure. At a certain size in the colloid molecule, or, as it might preferably be termed, 'solution aggregate,' the direct method also ceases to give measurable readings of osmotic pressure, but in all probability this is only due to a certain size of solution aggregate being attained which transcends the delicacy of the direct method, and if we possessed a delicate enough method of detection osmotic properties might still be found in the suspended colloidal masses.

Thus, it has recently been shown by Ross<sup>2</sup> that particles of such a size as mammalian blood corpuscles can travel upwards through a solid agar-agar jelly, against the action of gravity, and permeate the whole jelly. In such a case there can be no convection currents, and the movement is probably due to diffusion of the corpuscles into the jelly. But true diffusion, apart from convection currents, or directive movement of charged particles in a field of varying electrical potential which is here obviously excluded, can only be due to osmosis.

If the diffusive movement of crystalloids be due, as is generally accepted, to osmotic pressure differences, why should not diffusive movement of colloids be due to osmotic pressure differences?

All staining acts due to the penetrating of colloidal dyes are also evidences of the diffusibility, and hence of the osmotic pressure, of colloids.

As the colloidal particles of the dye become adsorbed, or chemically combined with the tissue or fabric being dyed, fresh

1. This term was introduced by Moore and Parker (*vide infra*) to express the physical constant that in the case of the colloid corresponds to the molecular weight of the crystalloid; the solution aggregate may consist of many or few chemical molecules, but for purposes of producing osmotic pressure each solution aggregate has the same effect as a dissolved molecule or ion.

2. *Brit. Med. Journ.*, Vol. I, 1906. p. 1027.

particles pass in from the solution outside on account of the lowered concentration of the dye within the tissue; but if the colloidal particles be supposed to exert no osmotic pressure there remains no reason why any particles should pass into the tissue, because there would exist no falling gradient of pressure urging them in that direction.

If a suspended or dissolved molecule or particle does not exert any osmotic pressure, there is no longer any reason why it should dissolve or be suspended (unless the specific gravity of the solvent is exactly the same as its own), nor any cause why the molecules or particles should distribute themselves uniformly throughout the solvent by a process of diffusion. By the formation of convection currents, or by mechanical shaking and admixture of solvent and particles, a *temporary* emulsion might be formed in absence of osmotic pressure, but the condition would be a temporary one only, the duration of which would depend upon the difference in specific gravity of particles and solvent, the size of the particles, the internal friction between particle and solvent, and other physical factors. The only active opposition to sedimentation is that arising from the osmotic pressure which produces diffusion from a region of high concentration to one of low concentration, and so equalises distribution of the dissolved (or suspended) substance throughout the whole of the volume of solvent.

While it thus necessarily follows that if osmotic pressure ceased to exist in the molecules or particles of a substance in solution or suspension, sedimentation must occur, the converse does not follow that because sedimentation does take place there exists no osmotic pressure. This condition may arise on account of the tendency of osmotic pressure to prevent sedimentation being overcome or outbalanced by the forces tending to produce sedimentation or separation into two phases.

At a certain concentration there is a balance or equilibrium between the two opposing forces, and at this point the solvent is just saturated with the solute or dissolved substance. If the concentration is less than that of the equilibrium point, then, if any undissolved

substance be present, the osmotic pressure tends to send it into solution until the equilibrium point is reached; if, on the other hand, the forces of cohesion and gravity outbalance the tendency to solution given by the osmotic pressure, then substance is thrown out of solution and sedimented until the equilibrium point is attained.

The fact that the osmotic pressure in the case of colloids or suspensions is low compared with that shown by crystalloids does not minimize the importance of its study, because that very circumstance, viz., large size of colloid molecule or solution aggregate, which renders the pressure low, provides the means of making the low pressure more effective by decreasing the permeability of any membrane containing the solution for the large molecule. So that osmotic pressure of colloids becomes apparent and is brought into action under conditions in which the greater osmotic pressure of crystalloids produces no effect. Further, the osmotic pressure of colloids is of especial importance in bio-chemistry because so much of the nutritive material of the living cell is presented to it in colloidal form, and so many of the normal and pathological products of cell activity are colloids. If such colloids possess no osmotic pressure then they can only be carried from one position to another by purely mechanical means, or by the activity of the cell, and the usual powerful aid to such processes given by the tendency to diffuse from a position of higher to one of lower concentration, will be entirely lacking.

It will, accordingly, be well to sift the supposed evidences against the possession of osmotic pressure by colloids before proceeding to a description of our experiments.

The first point is the large size of the colloid molecule, which is supposed by many to be so great and so nearly approaching to that of a mechanically suspended particle that the laws governing chemical molecules in solution cannot be expected to apply to it.

In reply to this objection it may be pointed out that the range of molecular weights in the case of typical crystalloids is much greater than that between the crystalloids with higher molecular weights and typical colloids. Thus the hydrogen ion, with a molecular weight of



1, and the cane sugar molecule, with a molecular weight of 342, have precisely the same effect in producing osmotic pressure, the great variation in the mass of the molecule causing not the slightest variation in the result. And bodies with even higher molecular weights, such as lecithin, with a molecular weight of 780, behave like true crystalloids in producing normal osmotic pressures. There is nothing inherently improbable, therefore, in a colloid, with a solution aggregate of 40,000 or 50,000, possessing a normal osmotic pressure corresponding to this higher value. The ratio between hydrogen ion and cane sugar molecule, where the law of osmotic pressure is admitted to hold rigorously, is 1 : 342, while that between cane sugar and serum albumen is 342 : 60,000 approximately, or 1 : 178; and for cane sugar to gelatine, which behaves as a typical colloid, it is 342 : 10,000, or 1 : 29. Accordingly, the range between typical colloid and typical crystalloid is shorter than that between different crystalloids.

A second argument against typical colloids possessing osmotic pressure is that the usual methods employed for estimating osmotic pressure, viz., lowering of freezing point or depression of vapour pressure (rise in boiling point), give negative results, or readings which lie within the limits of experimental error.

In order to make clear the relationship in the values to be expected in the case of determination of the solution aggregate of a colloid, (*a*) by an indirect method, such as determination of depression of freezing point, and (*b*) by a direct method, such as reading the osmotic pressure in millimetres of mercury, as shown by an osmometer like that described below, the following calculation may be set forth:—

Taking water as the solvent which interests us most, and supposing, for simplicity of calculation, we are dealing with a substance which is not ionized in solution, then the molecular depression of the freezing point is approximately  $18.4^{\circ}$  C., which means that the molecular weight in grams of the substance dissolved in 100 c.c. would lower the freezing point  $18.4^{\circ}$  C., or a normal solution containing the molecular weight in grams dissolved in a litre would give a lowering of freezing point of  $1.84^{\circ}$  C. Now when the molecular weight in grams occupies a volume of 22.33 litres the osmotic pressure



is one atmosphere, or 760 millimetres of mercury pressure. Hence, the normal solution containing the molecular weight in grams in 1 litre has an osmotic pressure of 22.33 atmospheres, or  $22.33 \times 760$  millimetres of mercury, and this corresponds to a lowering of freezing point of  $1.84^\circ \text{C.}$ , the calculation of the osmotic pressure due to a lowering of freezing point of  $0.001^\circ \text{C.}$  is simple from this relationship, it is :—

$$\Delta \text{ of } 0.001^\circ \text{C.} = \frac{22.33 \times 760}{1.84 \times 1000} = 9 \text{ mm. of mercury}$$

When it is remembered that the smallest reading of difference of freezing point that can be measured is 0.005, that is half of one of the smallest divisions on the usual Beckmann's thermometer, it follows that an osmotic pressure of 45 millimetres of mercury would escape detection by the freezing point method.

A similar result follows for the boiling point method, and it is obvious that the only method known for estimating osmotic pressures of this order, is the direct method by a membrane impermeable to the colloid.

Accordingly, the fact that freezing and boiling point methods give no reliable readings does not prove that colloids possess no osmotic pressure.

A third argument usually urged against the possession of osmotic pressure by colloids is that the readings of osmotic pressure shown in the osmometer by the direct method are due to the osmotic pressure of crystalloids present as impurities along with the colloids. This argument is most difficult to discuss, both from the theoretical and experimental points of view, because there is no doubt that the presence of inorganic matter, which would undoubtedly have to be regarded as crystalloidal if present by itself, along with colloid in the solution is responsible in some fashion for the production of the osmotic pressure. It is precisely in the case of those colloids which are closely associated in solution with crystalloids, that positive results are obtained by the direct method.

No readings of osmotic pressure by the direct method have ever been obtained by any observer in a solution entirely free from

inorganic salts. In those cases where inorganic salts are not closely associated with the colloid, such as starch solution (*vide infra*) no osmotic pressure is shown, and further, when colloid, such as proteid, is dissociated from salts, the osmotic pressure is decreased. Hence, the osmotic pressure which can be directly demonstrated by the osmometer in the case of colloidal solutions is closely associated, in some manner, with the presence of crystalloidal substances also present in the solution.

This is far, however, from saying that the colloids are not responsible for the production of the osmotic pressure, or that the osmotic pressure observed is due to crystalloids present as impurities which would give the pressure apart from the presence of the colloids.

The position is one requiring careful analysis, and consideration of the various experimental results, in order to determine the share played by crystalloid and colloid in the production of the osmotic pressure.

In the first place, there is abundant proof in the experimental evidence recorded by all observers on direct measurement of osmotic pressure in colloidal solutions, that the osmotic pressure is not due to any substance, colloidal or crystalloidal, which can diffuse even slowly through the membrane.

This is shown by the fact that the manometer, attached to the osmometer, rises at first rapidly and then more slowly to a maximum value, *and then remains stationary for days at that maximum value.*

This would not be the condition of affairs were the pressure due to any substance capable of diffusing through the membrane; then there would be a rise to a maximum value, followed by a fall as the concentrations of the substance diffusing through tended to become more nearly equal on the two sides of the membrane. Such a condition is realised when a diffusible substance, such as crystalloid alone, is placed in solution on one side of the membrane and pure solvent on the other side. Thus, with a one per cent. sodium chloride solution on one side and water on the other side, although the difference in real osmotic pressure of the two fluids is initially enormous, there is only a rise of a few millimetres in the first

few hours, which rapidly subsides to zero, and then analysis for chlorides on the two sides shows practically equal amounts. A more slowly diffusing crystalloid shows a slower and smaller rise and a slower fall, but there is never a maximum which is followed by a constant summit or plateau. With a membrane of parchment paper, such as was employed in our experiments, the equalisation was so rapid that even with sugar solutions no appreciable rise of pressure was obtained, and analysis at the end showed zero pressure and equal concentration in sugar on the two sides of the membrane.

The osmotic pressure readings obtained with colloids could not therefore be due to crystalloids not associated with colloids, because such crystalloids must have diffused through the paper membrane unless prevented from doing so by some association with the colloid either by adsorption or chemical combination.

Under such conditions the crystalloid must be regarded not as an impurity but as an integral constituent of the colloid molecule or solution aggregate, which confers on the aggregate the property of giving osmotic pressure in measurable amount. In the absence of the crystalloid the colloid molecules, or aggregates, are not stable in the solution, but run together to form solution aggregates so large that the osmotic pressure becomes too low for measurement.

The change in value of the solution aggregate when the amount of crystalloid is altered has been drawn attention to previously by Moore and Parker.<sup>1</sup>

These observers have shown that the osmotic pressure of the serum proteids changes, in the same sample of proteid, when the amount of diffusible salt present is changed, thus the same serum when in equilibrium against distilled water gave an osmotic pressure of 28.2 mm., as against 22.2 mm. with 0.7 per cent. sodium chloride, and 19.3 mm. with 1 per cent. sodium chloride, leading to values for the 'solution aggregate' of 40,383, 50,504, and 58,327 respectively. Still greater changes were observed when dilute alkali was allowed to act upon the serum, the osmotic pressure rising to about five times

1. *Amer. Journ. of Physiology*, 1902, Vol. VII, p. 261.

its value, and the solution aggregate falling approximately from 50,000 to 10,000.

These results show that the degree of association of the proteid to form 'solution aggregates' of varying size depends to a large extent upon the amount and nature of the crystalloids present, at the same time the colloid is absolutely necessary for the appearance of the osmotic pressure, the crystalloids alone upon one side as against pure solvent on the other producing no permanent pressure.

The permanent osmotic pressure readings obtainable, therefore, when colloids present in common solution with crystalloids are in equilibrium with crystalloids only in solution on the other side of the membrane of the osmometer, are due to colloid containing crystalloid in combination or adsorption. The function of the crystalloid is to keep the colloid in solution, and by its union with it prevent the formation of too large solution aggregates to give pressure readings, and the function of the colloid to render the crystalloid attached to it indiffusible so that it cannot pass the membrane, and hence gives rise to a pressure.

It does not follow that the presence of any particular crystalloid in co-solution with colloid, will necessarily give rise to an alteration in osmotic pressure. For this purpose it is necessary that the colloid and crystalloid must be capable of union in the form of combination or adsorption with one another. Thus it will be shown that starch, in distilled water, gives no measurable osmotic pressure, and that the addition of an equal concentration of sodium chloride on the two sides of the membrane has no effect in producing an osmotic pressure, but, on the other hand, as pointed out above, alteration of the concentration of sodium chloride present along with the serum proteid at once causes an alteration in the osmotic pressure.

It is clear, therefore, that in the case of those colloids which unite with crystalloids when in common solution that the crystalloids are not to be regarded as impurities, but as essential to the particular condition in solution of the colloid under which the osmotic properties are being measured, and that the osmotic pressure is due to the union



of crystalloid and colloid which preserves the solution aggregate of which the osmotic pressure is being determined.

The first definite measurements of osmotic pressure of colloids, by the osmometric method, were made by Starling,<sup>1</sup> who determined the osmotic pressure of serum, using a gelatine membrane, constructed between layers of peritoneal membrane.

Moore and Parker<sup>2</sup> published, in 1902, observations on the direct measurement of osmotic pressure with egg-albumen in native condition and after prolonged dialysis, serum-proteid and alkali-albumen formed from serum-proteid, and solutions of various soaps. These observers used parchment paper as a membrane, and a bell-shaped osmometer, and whilst criticising Starling's applications of the osmotic pressure of the serum proteids to the problems of lymph absorption and glomerular activity in the kidney, confirmed the experimental results obtained by Starling that the serum proteids possessed an osmotic pressure.

Moore and Parker introduced in their paper the term 'solution aggregate' for the aggregation of chemical molecules which gives rise to the unit of the colloid in solution corresponding to the chemical molecule of the crystalloid in solution. They showed that the osmotic pressure of a colloidal solution depended upon the value of this aggregate, and that in the case of dilute solutions of the sodium soaps, the value of the solution aggregate varied between thirty and sixty times the value of the molecular weight. As stated above, the value of the solution aggregate with the same colloid was shown to vary with the amount of crystalloid present. The effect of such variation in physical aggregation of molecules of proteid upon protoplasm formation, and in the building up of the minute structures of the cell, was considered, and it was pointed out that the activities of the colloid would vary with the degree of aggregation.

1. *Science Progress*, April, 1896; *Journ. of Physiology*, 1896, Vol. XIX, p. 312; 1899, Vol. XXIV, p. 317; Schäfer's, *Text-book of Physiology*, Vol. I, p. 307.

2. *Amer. Jour. of Physiology*, 1902, Vol. VII, p. 261.

E. Waymouth Reid<sup>1</sup>, in a paper, published in 1904, on the osmotic pressure of solutions containing native proteids, arrived at the conclusion that the pressures observed by Starling, and by Moore and Parker, were not due to the proteids, but to certain other substances recoverable from the washings of the proteids after precipitation with neutral saline ( $\text{Am}_2\text{SO}_4$ ).

The nature of these substances upon which, according to Reid, the osmotic pressure depends was not determined at the time, nor have been since, but it was shown that alone they did not produce pressure, but only when proteid was also present, or a membrane used which had been used previously with proteid.

Reid was led to the conclusion that the osmotic pressure shown by serum was not due to the proteids by the observation that the osmotic pressure was not exactly proportional to the amount of proteid

1. *Journ. of Physiology*, Vol. XXXI, 1904, p. 438.

[Note by B. Moore.—I desire to take this opportunity of replying briefly to certain criticisms made by Reid, in the above paper, upon the experimental methods used by Parker and myself in our former work, especially as some of them would also apply to the present experiments. Reid objects to the results on account of the osmometer used by us not having been stirred, but we expressly point out in our paper that the natural process of diffusion and differences in specific gravity in the bell osmometer acts as a most excellent stirrer, this being one of the chief advantages of that form of osmometer, as also of the one used in the present research, in this way we were able to dispense with the 80,000 daily mechanical stirrings used by Reid, and the analysis, mentioned on p. 276 of our paper, shows how complete the equilibrium was upon the two sides at the end of the experiment, the amounts of chloride being practically equal, so that it is difficult to see how stirring would have altered the results.

It is objected that our membrane being impermeable to soaps, the presence of soaps in the blood serum seriously invalidates the conclusion that the pressures observed are due to the proteids, but collateral evidence in our paper clearly shows that the osmotic pressure due to the traces of soaps present in blood serum could not appreciably affect the results, since it is stated by us that as much soap as one-half per cent. only causes a pressure of 6.5 mm. of mercury.

It is suggested by Reid that, as the duration of each experiment is not given, one cannot judge whether sufficient time was allowed for equalization of substances in solution (and to which the membrane was permeable) on the two sides of the membrane before the final reading of pressure was taken, and it is further suggested that the lower readings when sodic-chloride was used as outer fluid instead of distilled water, and where 1 % sodic-chloride replaced 0.7 % sodic-chloride solution, with one and the same sample, are internal evidence of the readings having been made before equilibrium had been attained. Considering the nature of the experiment which is being carried out, namely, that of recording the final value of a pressure which rises from zero, it would seem scarcely necessary to state that readings were continued until a steady pressure was attained; but, as it appears to be necessary, I may now state that the observations were continued until the pressure had been steady for two or three days. Further, in a note to our table, p. 276, we point out that with our osmometer a difference of 1 % of sodic-chloride on the two sides gave, at the maximum point of pressure in the process of equalization, only 4 mm. of mercury, and that the greatest difference of concentration of sodic-chloride on the two sides at the end of an experiment never exceeded one-twentieth of this amount, as determined by analysis, so that pressure due to difference in concentration of diffusible crystalloids might, we thought, be neglected. Regarding the differences in osmotic pressure when the proteid is in distilled water, 0.7 % saline and 1 % saline respectively, we expressly treat this at considerable length in our discussion of the results as an important point showing difference in aggregation of the proteid under these conditions.

It is this same alteration of state of aggregation of proteid due to lengthy manipulation, and removal of the natural alkali and salts of the serum, which appears to me to be the explanation of the loss of osmotic pressure observed by Professor Reid in his own experiments, and not the removal of hypothetical organic substances other than proteids from the serum.

present in the serum, and that after many washings of the precipitated proteid with neutral salts, such as ammonium sulphate, or by sodium chloride and acetic acid solution, and re-dissolving the proteid, a solution could be obtained which showed no osmotic pressure, and finally, that by adding the dialysed washings to such osmotic pressure free solutions, an osmotic pressure could be once more obtained.

In our opinion, these facts are capable of explanation otherwise than on the supposition that the serum proteids do not give an osmotic pressure.

In the first place, the fact that the osmotic pressure is not absolutely proportional to the amount of proteid present, is probably due to the fact already mentioned above, that the pressure depends not alone upon the amount of the colloid, but upon its degree of aggregation in solution, and this, in turn, upon the salts of the serum and its reaction, so that the proteid of the serum may be responsible for the pressure without there being an absolute linear proportionality in different samples of serum.

Secondly, in the prolonged process of precipitation and washing used by Reid, the serum proteid is removed from all the alkali and all the neutral salts with which it is combined or adsorbed in the natural condition, so that it is scarcely to be wondered at that its state of aggregation changes, and that it finally does not give a readable osmotic pressure.

Finally, with regard to the production of osmotic pressure once more on adding the dialysate of the first washing, it may be remarked that the pressures observed, of three to five mm. of mercury, are too small to inspire confidence in any deduction from them, and, secondly, that even then such readings are only obtained when the pressure free proteid is added to the dialysed washings, and that the true explanation of the small result obtained may be a partial return of the proteid to a less state of aggregation forming an approach to its natural condition in the serum.

More recently, Reid<sup>1</sup> has published experiments carried out

1. *Journ. of Physiology*, 1905, Vol. XXXIII, p. 12.

with haemoglobin solutions, and finds that this proteid does possess a genuine osmotic pressure, so that it is, according to him, in 'true solution,' while serum proteids are not. It would be interesting to test whether haemoglobin also loses its osmotic pressure after being precipitated and many times washed with ammonium sulphate solution, and if there was a partial restoration of osmotic pressure on dissolving the haemoglobin in the dialysed washings. Until this laborious process has been carried out, there is little logical basis for believing in a difference in character between the osmotic pressure of serum proteids and that of haemoglobin.

To sum up the experimental evidence hitherto published regarding osmotic pressure of colloids, osmotic pressure has only been recorded in solutions of colloids which are capable of adsorption with crystalloids, or of forming combinations with crystalloids. The pressure is not, however, due to crystalloids alone, for it is only found when colloid impermeable to the membrane is present, and the pressure remains permanent after all diffusible colloids have equalized on the two sides of the membrane. The pressure varies with the same amount of colloid, according to the state of aggregation of the colloid, and this can be altered according to the amount and character of the crystalloid present in the solution.

There is no evidence, save the failure of the direct osmometric method to give readings, that osmotic properties entirely cease whatever the size of the aggregate, and the failure of the method may be merely due to the low osmotic pressure given by such huge aggregates, for the fact of diffusion occurring is *prima facie* evidence in favour of the existence of an osmotic pressure if we possessed delicate enough methods to detect it.

#### EXPERIMENTAL METHODS

The osmometer<sup>1</sup> used for the experiments is illustrated by the accompanying sectional drawing (Fig. 1) and photograph (Fig. 2).

1. The construction of the osmometer, and preliminary results, were described in a short paper read at the Southport Meeting of the British Association, 1903. 'A new form of osmometer for direct determination of osmotic pressure of Colloids,' by B. Moore.



The drawing shows the osmometer alone in section, and the photograph the manner in which it is connected up and joined to the manometer by thick walled rubber tubing.

When it was desired to work at a constant temperature different from that of the laboratory, the osmometer was immersed in a water bath kept at the desired temperature by means of a thermo-regulator.

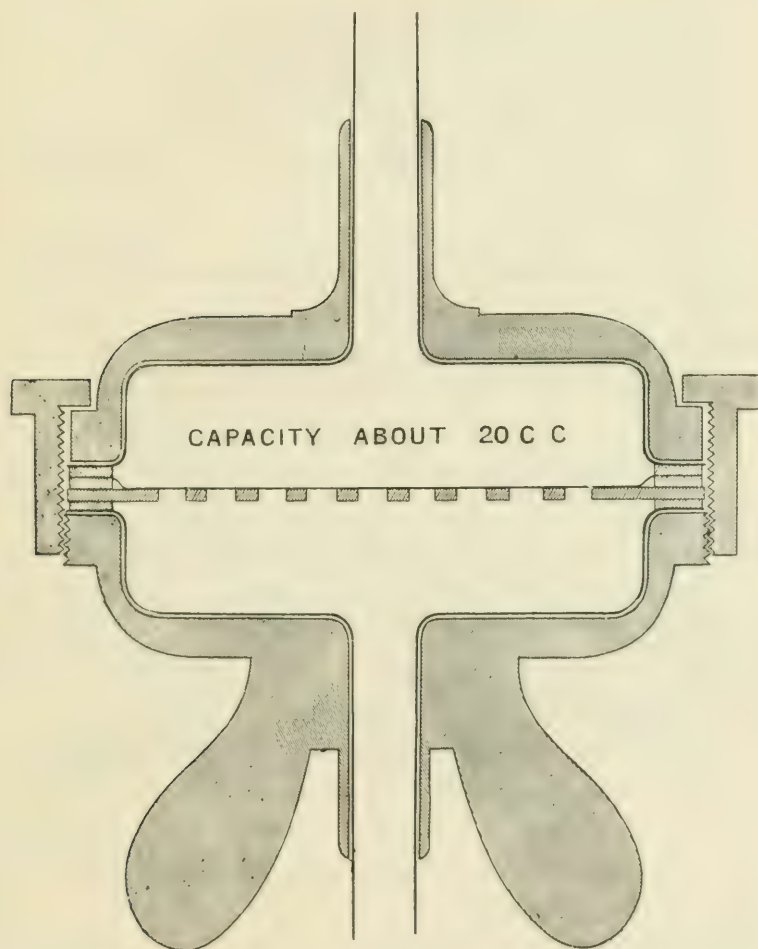


FIG. 1. Sectional view of Osmometer magnified  $1\frac{1}{2}$  times.

The osmometer consisted of platinum linings, or capsules, supported and held in apposition by thick walled brass chambers.

Each half of the platinum lining was the same in construction, being a circular shallow capsule with a platinum tube leading away from its base and projecting beyond a similar, but larger, tube in the thick walled brass case. Around the rim of each platinum capsule was a flange, which faced over the similar flat flange shown on the outer brass case. A thick platinum grid came, when the apparatus was screwed together, between the mouths of the two capsules, and served to support the membrane of parchment paper and prevent it from sagging as it came under the osmotic pressure. As the drawing is placed, the upper chamber contained the colloidal solution and the lower the fluid used against the colloidal solution and containing no colloid.

Three circular rings of thin soft rubber were arranged, one between the lower platinum flange and the grid, one between the grid and the parchment paper membrane, and one between the membrane and the upper platinum flange. The platinum grid and upper case were prevented from turning round, as the apparatus was being screwed together, by means of two small brass pins, not shown in the section, which were let in at opposite ends of a diameter of the lower thick casing close to the screw thread; these projected and caught two recesses cut in the edge of the platinum grid, and also corresponding recesses in the upper case. This precaution is absolutely necessary, otherwise, the turning round which occurs on screwing up tears up the membrane and rubber rings, and invariably causes leakage.

The osmometer is screwed together by means of a male screw on the lower casing and a female screw on a circular joining piece of thick metal possessing a flange, which fits against a flange on the upper case as shown in the drawing. The thumb hold on the lower case enables the whole to be tightly screwed together.

The capacity of each chamber was about 20 c.c., the diameter 5 cm., and the depth 1 cm.; so that a large surface for diffusion and rapid equalization of crystalloids was offered, relatively, to the volumes of the chambers.

In fitting up the osmometer a circle of parchment paper, the

exact size of the platinum grid, is cut out from a sheet, thoroughly wetted, and applied to the grid, which is then placed in position and the apparatus screwed tight. Any leakage is then tested for by putting the osmometer in a vessel of water, blowing air in, and observing whether any air bubbles appear. In order to prevent blowing the

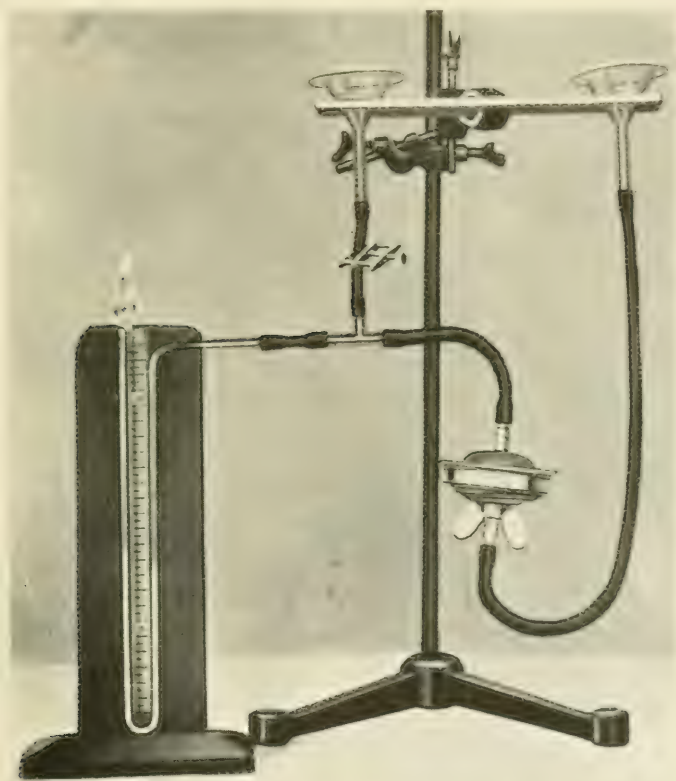


FIG. 2. Photograph of osmometer fitted up and joined to manometer.

membrane away from the platinum grid, the two outlet tubes of platinum may be joined, by rubber tubing, to a glass T piece, and blown up together, as any leakage is usually to the outside and not from one chamber to the other, good parchment paper in sheet being remarkably free from imperfections.

The next step is the filling of the osmometer. The chamber on the upper, or colloid, side is first filled with the solution, in which the pressure of colloid is to be measured, by means of a narrow tubed pipette, and the proximal limb of the mercurial manometer, which is of narrow bore, as also the connections and funnel on the manometer side (see Fig. 2) having been previously filled with the solution, or with oil, and clipped off at the end to be connected to the osmometer, the osmometer and tubing are joined. Care should be taken to avoid inclusion of air in making the connection, as far as possible, but a bubble or two of air only slightly delays the time of reaching a steady pressure reading. By care in manipulation, any air accidentally included can be got into the vertical tube, and so out by the funnel. The screw clip on the vertical tube is at first left open. The osmometer is then turned other side up, and the other chamber of the osmometer is similarly filled with the non-colloidal solution, which is to come into equilibrium with the colloidal solution, save for content in colloid. This chamber is, in turn, joined up to its tubing and funnel, which have been previously filled with the same fluid as the chamber, and the osmometer is turned so that this chamber lies lowest down. The two funnels are supported at the same level, and the level of the fluid in them is made the same. The clip is then left open on the colloid side for about half-an-hour to let the whole come, approximately, into equilibrium, then the screw clip is closed, shutting off communication with the funnel on the colloid side, but leaving open the communication with the manometer. A zero reading of pressure is now made on the manometer, and readings are continued at intervals, as seen in the tables of experiments, until a steady pressure is attained, as shown by constancy of reading.

It is important, as a saving of time, that the chamber on the colloid side be filled first, joined up as above described, and clipped on the rubber tubing just beyond the platinum tube, so as to get the pressure from osmometer to funnel upon the membrane before the other side is filled, for if the other side be filled first, the membrane is pressed away from the platinum grid, and it takes a considerable time before diffusion of water through the membrane increases the



volume in the upper chamber, and forces the membrane tight against the grid, until this takes place, the pressure does not begin to rise in the upper, or colloidal, chamber.

## EXPERIMENTAL RESULTS

### *Experiments with Gelatine Solutions*

A great advantage of the osmometer described above is that, on account of the unalterable nature of the parchment paper membrane, and of the platinum walls of the chamber, solutions of colloids which do not undergo heat coagulation on boiling, such as gelatine or starch, can be completely sterilized by raising the chamber and its contents to nearly  $100^{\circ}\text{C}$ , and keeping at that temperature for some minutes. The advantage of this is that an experiment, with such a substance as gelatine, can be continued for months if necessary, and the point in this way settled as to whether the observed osmotic pressure be due to some slowly diffusing crystalloid present as an impurity along with the colloid. If this were the case, equalization must, in the end, occur, and the pressure fall to zero.

In the experiment with gelatine recorded below, the same quantity of solution of gelatine was used throughout, during a period of two months and twenty-five days, or eighty-five days (Nov. 4th, 1902, till Jan. 28th, 1903). When taken out of the osmometer at the end of this long period it was, to all outward appearance, the same as when it was put in, forming a stiff, clear jelly, which melted at  $24$  to  $25^{\circ}\text{C}$ , began to re-set at  $22.5^{\circ}\text{C}$ , and was completely set at  $21^{\circ}\text{C}$ . The fluid on the other, or water, side of the membrane was a clear watery fluid, which gave a negative result on testing for protein by the biuret, acetic acid and ferrocyanide, and tri-chloroacetic acid test. It contained 0.074 per cent. of organic matter, and 0.032 per cent. of ash.

The gelatine solution, which had been made up at the beginning of the experiment lasting nearly three months to contain 10 per cent

of gelatine, contained, at the end, 9.56 per cent. of organic matter destroyed by incineration, and 0.17 per cent. of inorganic ash.<sup>1</sup>

As soon after the commencement of the experiment as equilibrium had been reached, the gelatine solution showed an osmotic pressure of 74 mm. of mercury at 31° C. (Nov. 11th, 1902); near the end of the experiment, over two months later, during all of which time dialysis and equalization of any diffusible crystalloids present had been going on, it showed (on Jan. 27th, 1903) a pressure of 70 mm. at 26° C.

This constancy of the pressure, and its persistency after such a period, show that it is something existing when equilibrium has been attained, and not a passing phase due to slowly diffusing crystalloids, and since, in the absence of the gelatine, the soluble crystalloids would have equalized on the two sides in the course of a few days, we regard the experiment as a crucial one, showing that colloidal solutions can exhibit osmotic pressure.

The experiments with gelatine were also used to determine, as far as possible, the effect of temperature on osmotic pressure of colloids; gelatine, on account of its not coagulating by heat, being a suitable colloid for this purpose. The results show clearly that the osmotic pressure rises with the temperature. The rise is not proportional to the absolute temperature, occurring more rapidly, but this may arise from partial dissociation of the colloidal molecules, or solution aggregates, of the gelatine as the temperature rises.

When the temperature is lowered, so that it lies just above the point at which a jelly is formed, there is no sudden fall in pressure, but if it is kept for some days at this temperature a very marked continuous fall is obtained, showing that, in the neighbourhood of the temperature of formation of the hydrogel, a rapid aggregation to form much larger solution aggregates occurs. At temperatures just below that of formation of the hydrogel it

1. This amount of ash, calculated on the percentage of dried gelatine (organic matter), amounts to 1.74 per cent. of inorganic constituent. The long duration of dialysis in a chamber specially built for rapid equalization, and the difference in percentages of ash on the two sides, at the end, viz., 0.032 and 0.170 per cent. show that this inorganic matter is not to be regarded as an impurity in the gelatine, but as an intrinsic part in combination or adsorption with it.

is difficult, on account of some of the experimental difficulties introduced by the setting of the solution, to determine whether a low osmotic pressure exists or not, but in a portion of the experiment in which the upper surface of the gelatine was covered by oil, which also covered the connections to the manometer, it was found that a low pressure of about 10 mm. was still obtained at about  $2^{\circ}$  C. below the setting point of the solution.

A further fall in the temperature, to about  $10^{\circ}$  C. below the setting point of the solution, caused complete disappearance of all pressure.

After the gelatine solution has been kept for a short time at a higher temperature ( $70^{\circ}$  to  $80^{\circ}$  C.) it not only shows a high pressure out of linear proportion to the absolute temperature, but, after the temperature is allowed to fall again, much higher readings than before are obtained at the lower temperatures, and this continues for some days *after which there appears to set in a recurrence to the former values.*

If the heating to the higher temperatures ( $80^{\circ}$  to  $90^{\circ}$  C.) be continued for a longer period (see Experiment ii) a *permanent* condition of change with a higher osmotic pressure is produced, and with prolonged heating, as is well known, the gelatine is so permanently altered that it no longer sets on cooling, and, at the same time, protein derivatives are formed which pass through the membrane, and give a solution on the other side, showing the biuret and other protein tests.

*(Commenced on November 4th, 1902, at 10.30 a.m.)*

*Experiment I.*—Filled the osmometer with a ten per cent. solution of best French gelatine as above described on the upper side, the platinum tube leading from the platinum capsule and the connections to the osmometer were filled with pure neutral olive oil to prevent solidification of gelatine in these connections. In the under platinum capsule was distilled water.

FIRST PERIOD						
		Time from commencement of experiment		Osmotic Pressure in mm. of mercury	Temperature in degrees Centigrade	
		Days	Hours	Mm.		
November 4th	...	0	0	...	0	15°
		1	8	...	57	23°
		2	0	...	64	25°
		3	0	...	82	29°
		4	0	...	90	40°
		8	0	...	80	32°
		9	0	...	85	36°
		10	0	...	88	40°
		14	8	...	77	30°
		15	0	...	78	27°
November 21st	...	17	0	...	64	22°

The temperature was allowed to fall, after keeping for some days at about 20° C., down to laboratory temperature (13° to 14° C.), the osmotic pressure falling as a result to zero. During the long intervals equalization of all diffusible crystalloid must have been going on.

On December 6th (i.e., 32 days after starting) the osmometer was disconnected and opened. On the water side a perfectly clear watery fluid was found, which showed no signs of any decomposition. Analysis showed that this contained 0.132 per cent. of organic matter and 0.065 per cent. of fixed ash.

The gelatine side contained a clear, transparent, colourless jelly which filled the whole of this capsule of the osmometer and the proximal half of the attached platinum tube, the india-rubber connecting tube being filled with oil. The jelly melted at about 25° C.

Since the jelly was so unchanged it was not immediately analysed, but used for a further continuation of the experiment.

#### SECOND PERIOD

The same identical quantity of gelatine was used again, being introduced into the upper capsule of the osmometer, covered with oil, and joined up as before with connections also filled with oil to the manometer; the lower capsule was filled with distilled water. Before introduction into the capsule of the osmometer the gelatine solution was heated for five minutes to 95° C. in order to sterilize. After the osmometer had been filled on both sides as above described and connected up, it was immersed in a large beaker in a constant level water bath, and the bath and osmometer raised to 85° C., and kept for 30 minutes at about that temperature, the clip on the T piece on the manometer side being left open. The temperature was then allowed to fall to 40° C., and regulated to about that temperature by a thermo-regulator. A zero reading of pressure was taken and the clip then closed on the T piece on the manometer side, so as to begin to allow osmotic pressure to develop.



	Time from commencement of this period		Osmotic Pressure in mm. of mercury	Temperature in degrees Centigrade
	Days	Hours		
December 8th, 1902,	0	0	...	0
5 p.m., 32 days after	0	17	...	100
first commencement	1	0	...	107
of experiment	1	18	...	107
	2	0	...	106
	2	19	...	106
	3	0	...	105
	3	17	...	104
	4	1	...	106
	4	22	...	106
	7	1	...	104
	9	1	...	98
	10	0	...	90
	11	17	...	82
	13	17	...	78
49 days from first commencement	14	18	...	72

The pressures recorded during this period are considerably higher than those obtained at corresponding temperatures during the first period. The only explanation we can suggest is that the high temperature of 85° C., to which the gelatine solution was subjected at the commencement of the period, caused a separation of the colloid aggregates to smaller dimensions, and that it takes a prolonged period for re-association to occur when the solution is allowed to cool but still kept considerably above the setting point. That the gelatine was not appreciably altered permanently is shown, first, by the fact that there was a reversion to the old values after the solution was allowed to set and then just raised to 40° C. (see Period III), and, secondly, because at the end of the entire experiment, about a month later, the gelatine was found still to form a clear stiff jelly, melting at almost the same temperature as at the commencement of the experiment.

On December 30th, 1902 (56 days from commencement of experiment), the pressure was allowed to drop to zero by opening the clip on the manometer side, and the temperature was kept 3° to 4° C. below the setting point of the jelly in order to see if an osmotic pressure manifested itself under these conditions. The pressure at first rose to about 20 mm., and then slowly fell to about 9 mm. The experiment, therefore, appeared to indicate a low pressure even in the hydrogel form, but it is difficult to be certain of pressure transmission in the hydrogel form of the gelatine, and hence this was pushed no further.

## THIRD PERIOD

The osmometer which had been left at laboratory temperature ( $11^{\circ}$  to  $13^{\circ}$  C.) from January 8th to 10th, 1903, showed zero pressure. The temperature was now raised and regulated at a level just more than sufficient to melt the hydrogel, and when the pressure had become approximately constant readings were taken.

	Time from commencement of this period		Osmotic Pressure in mm. of mercury	Temperature in degrees Centigrade
	Days	Hours		
January 15th	5	7	67	$23^{\circ}$
Time from beginning,	6	0	72	$25^{\circ}$
72 days	7	1	83	$28^{\circ}$
	9	0	86	$30^{\circ}$
	11	7	94	$39^{\circ}$
	12	8	94	$40^{\circ}$
	14	0	70	$21^{\circ}$
84th day from beginning	17	0	70	$26^{\circ}$

It may be pointed out that these pressures lie at the corresponding temperatures much closer to those of the first period than do the readings of the second period to either. This shows that the disintegrating effect produced upon the colloid aggregates by the heating to  $85^{\circ}$  C., and which remained in part for a long time after the temperature was reduced, had finally been recovered from either as a result of the hydrogel having been allowed to form or by the prolonged time which elapsed after the heating.

On January 28th, 1903 (85 days from beginning), the osmometer was finally opened for the second time, and the fluids on the two sides examined and analysed for total organic matter and ash.

On the water side there was found a clear, colourless, watery fluid, which gave a negative result with the biuret, acetic acid and ferrocyanide, and tri-chloroacetic acid tests for protein. Ten c.c. were evaporated down for analysis, and gave 0.074 per cent. of organic matter and 0.032 per cent. of ash.

On the gelatine side there was a stiff clear jelly, which became quite fluid at  $24^{\circ}$  to  $25^{\circ}$  C., and began to reset at  $22.5^{\circ}$  C., and was completely set at  $21.5^{\circ}$  C. A weighed amount of this jelly (22.8 grms.) was evaporated down to a constant weight on a steam bath, and yielded 2.1812 grms. of dried solid or 9.56 per cent. (the solution was made originally 10 per cent.), and after incineration the ash was found to be 0.169 per cent.

The points that may be specially noted in the experiment are the persistency and constancy of the osmotic pressure at the end of such a prolonged period. The greater rise with temperature of the osmotic pressure than is linearly proportional to the absolute

temperature showing a diminution in the value of the solution aggregate with rising temperature. The effect of a higher temperature in causing a sustained increase in the osmotic pressure, which only reverts approximately to its old level after many days, and the re-formation of the hydrogel condition. And, lastly, the important point, that even after such a long period for equalization of diffusible crystalloids the amount of ash is entirely different on the two sides, showing that a combination, or, if the term be preferable, adsorbate, exists between gelatine and inorganic constituent. Hence the inorganic constituent is an essential part of the gelatine under the conditions in which it exists, and not an admixed impurity.

*Experiment II.*—In this experiment, which in point of time was carried out before the preceding one, the osmotic pressure was observed throughout a wider range of temperature, and a marked increase was found at temperatures above 70° C., but the prolonged heating at the higher temperatures permanently altered the gelatine so that at the end a thin fluid which did not gelatinize on cooling was found on the gelatine side. The fluids on both sides of the membrane at the end gave the biuret test with a colour between purple and pink, but the depth of tint was much greater in the case of the fluid on the gelatine side.

The experiment is, nevertheless, of interest in showing the great rise with temperature, and the persistence of a higher pressure on re-cooling similar to that shown in Experiment I.

	Time from commencement of experiment		Osmotic Pressure in mm. of mercury	Temperature in degrees Centigrade
	Days	Hours		
October 20th, 1902 ...	3	3	107	51°
	6	3	116	61°
	7	0	126	71°
	8	1	148	80°
	9	0	158	91°
	10	0	142	80°
	11	0	138	7°
	11	6	134	60°
	11	22	134	47°
	12	3	122	33°
	12	6	114	21°
	13	5	98	16°

In carrying out this experiment the pressure was allowed to become steady, and then, by altering the thermo-regulator, the temperature was raised approximately 10° C. at each interval in the first half of the experiment, and lowered 10° C. in the second half of

the experiment. The intervals of time are made shorter between the readings in the latter portion of the experiments, but the changes in pressure set down are not due to the instrument acting as a thermometer, but to actual variations in the osmotic pressure. This is shown by the fact that with rising temperatures the first initial change occurring within the first half hour of the alteration in temperature is larger than the true change in osmotic pressure, and is followed by a rapid fall to the correct osmotic pressure. Similarly with the temperature being lowered there is at first a sharp drop, due to the osmometer acting at first as a thermometer, followed rapidly by a rise as diffusion equalizes and shows the true osmotic pressure by a steady manometric reading.

For brevity these intermediate readings are not given, but the following may serve as a sample :—

Time		Osmotic Pressure			Temperature
Days	Hours				
6	3	...	116	...	61°
Temperature raised to 70° C.					
6	6	(initial reading)	138	...	70°
7	0	(final reading)	126	...	71°
10	0	...	142	...	80°
Temperature allowed to fall to 70° C.					
10	1	(initial reading)	118	...	70°
11	0	(final reading)	138	...	70°

#### EXPERIMENTS WITH STARCH SOLUTIONS.

The experiments described above appear to us to prove conclusively that one colloid, gelatine, possesses in solution osmotic pressure, those now to be described show equally clearly that another colloid, starch, has no osmotic pressure within the limits measurable by the direct method, not even a single millimetre of constant pressure having been obtained throughout our work.

The starch used was potato starch, prepared in the laboratory, and made as pure as possible, by decantation from distilled water and thorough washing with alcohol and ether, from all soluble matter.

*Experiment I.*—A starch mucilage of approximately 1 per cent. concentration was prepared in the usual way by boiling with distilled water; this was introduced into the upper chamber of the osmometer as already described, and the lower chamber was filled with distilled water.



	Time			Osmotic Pressure in mm. of mercury		Temperature in degrees Centigrade
	Days	Hours				
March 15th, 1904 ...	0	0	...	2	...	13°
	0	15	...	0	...	12°
	1	0	...	1	...	15½°
	1	15	...	0	...	15°
	2	15	...	0	...	13°
	3	0	...	0	...	15°
	3	15	...	0	...	14°
	5	15	...	0	...	14°
	6	15	...	0	...	14°
March 25th, 1904 ...	9	15	...	0	...	14°

Examination of the fluid on the water side showed at the end of the experiment not a trace of starch. The mucilage on the other side was dried in a steam bath to a constant weight, and was found to contain 0.89 per cent. of starch.

*Experiment II.*—Since it is impossible to introduce a stronger starch mucilage than 1 per cent. through the narrow platinum tube of the osmometric chamber, the expedient was resorted to of making the stronger mucilages of starch within the chamber itself. For this purpose a certain weighed amount of the pure potato starch was introduced into the upper chamber in a dried condition, a sufficient amount of distilled water was introduced to fill up the chamber, the lower chamber was filled with distilled water, and the osmometer, after being attached to its connections, was heated up in a large beaker to a temperature sufficient to break up the starch granules and form a mucilage. Then, at the end, a weighed amount of the thick mucilage was taken, the water evaporated off in a steam bath, and the starch weighed when the weight had become constant. In this way the percentage of starch in the mucilage was estimated.

February 9th, 1903.—One gram of dry potato starch was weighed out and introduced into the pressure side of the osmometer, which was filled with distilled water. Distilled water was filled into the other side. The whole was heated up to 60° C. and left over the night.

February 10th, 10.30 a.m.—Pressure at zero, temperature 15.9° C., heated up to 90° C.; manometer at first rose from osmometer tending to act as thermometer to 12 mm., but fell back within an hour to zero.

	Time			Osmotic Pressure in mm. of mercury		Temperature in degrees Centigrade
	Days	Hours				
February 10th, 1903, at 6 p.m.	1	0	...	-0.2	...	60° C.
	1	16	...	0.0	...	16° C.
	1	18	...	+0.8	...	83° C.
	1	20	...	-0.2	...	82° C.
	2	0	...	-0.2	...	83° C.
	2	1	...	2	...	15° C.

The experiment was then stopped, communicability of fluid pressure throughout the apparatus having been proved by the fact that a quick oscillation of the mercury in the manometer occurred when the rubber tubing on either side was compressed with the fingers. The osmometer was emptied and the contents on the two sides examined.

On the distilled water side there was found a colourless mobile fluid, as clear as water, which gave no colour reaction with iodine and no reduction with alkaline copper sulphate.

On the starch side there was found partially a thin translucent fluid and partially a stiff jelly, which retained the shape of the osmometer on removing, but was easily broken to pieces. The mixture was heated in a vessel, while it was stirred with a thermometer, it melted into a homogenous viscid mucilage containing no visible solid particles at  $70^{\circ}\text{C.}$ , and remained a viscous mass until  $100^{\circ}\text{C.}$  was reached. The percentage of starch determined as in the preceding experiment was 5.56 per cent.

*Experiment III.*—Starch mucilage in saline (1 per cent. sodium chloride solution) first against saline, secondly against distilled water.

Both chambers of the osmometer were well washed out several times with 1 per cent. sodium chloride solution after a new membrane had been fitted in.

One gram of dry starch was then introduced into the upper chamber, which was afterwards filled with the 1 per cent. solution of sodium chloride. The lower chamber was filled with the 1 per cent. sodium chloride solution only.

The whole was then heated to  $85^{\circ}\text{C.}$ , the screw clip to the funnel on the starch side screwed up, leaving the starch chamber connected to the mercurial manometer, and the experiment commenced.

	Time		Osmotic Pressure in mm. of mercury	Temperature in degrees Centigrade
	Days	Hours		
February 14th, 1903 ...	0	0	...	$82^{\circ}\text{C.}$
	0	2	...	$82^{\circ}\text{C.}$
	1	21	...	$14^{\circ}\text{C.}$
	2	0	...	$82^{\circ}\text{C.}$
	2	2	...	$84^{\circ}\text{C.}$
	2	5	...	$85^{\circ}\text{C.}$
	2	21	...	$13^{\circ}\text{C.}$

The salt solution was now removed from the lower chamber, and after washing out several times it was filled with distilled water, and the osmometer set in action again.

	Time		Osmotic Pressure in mm. of mercury	Temperature in degrees Centigrade
	Days	Hours		
	3	0	...	$13^{\circ}\text{C.}$
	3	5	...	$84^{\circ}\text{C.}$
	3	22	...	$13^{\circ}\text{C.}$
	4	0	...	$84^{\circ}\text{C.}$
	4	4	...	$84^{\circ}\text{C.}$

To make certain that the zero pressure obtained throughout<sup>1</sup> this experiment was really a true indication, and that the membrane was acting properly, the pressure in the starch-containing chamber was now artificially raised by the introduction of 1 per cent. saline under pressure from the funnel, and the clip screwed down. The results in the following table show that the pressure gradually fell to zero, indicating that this is the true equilibrium point, as found in the previous parts of the experiment:—

	Time		Pressure (artificially caused initially) in mm. of mercury	Temperature in degrees Centigrade
	Days	Hours		
February 18th, 1903,	0	0	...	43
4.15 p.m.	0	0 $\frac{1}{2}$	...	35
	0	1 $\frac{1}{2}$	...	20.2
	0	2	...	9
	0	18	...	0
	0	20	...	0
				81
				81
				81
				81
				14°
				81°

Examination of the fluids at the end gave similar results to preceding experiment. With the same membrane the 1 per cent. saline solution was introduced on one side, and distilled water on the other. During fourteen days there was no measurable rise in the osmometer, and at the end of the time the concentrations in sodium chloride were practically equal on the two sides. The membrane was examined carefully, and found quite sound and free from holes.

*Experiment III.*—One gram of dried starch placed in osmometer, March 24th, 1904, 10 a.m.; both sides filled with distilled water; kept at 100° C. for some time, then allowed to cool, and experiment started at laboratory temperatures. This experiment may be regarded as one with a mucilage saturated with starch at the temperatures stated, and at the end only the thinner portion was taken for determination of percentage of starch, and not the whole of the material in the osmometric chamber as in Experiment II of this section.

	Time		Pressure in mm. of mercury <sup>2</sup>	Temperature in degrees Centigrade
	Days	Hours		
March 24th, 1904,	0	0	...	6
6 a.m.	0	15	...	6
	1	15	...	2
	3	15	...	0
	4	16	...	0
				17
				17
				16
				16
				16

The fluid in the water chamber of the osmometer was free from starch; that on the starch side contained a thick jelly and a thin, slightly translucent fluid. In this case

1. The small fraction of a millimetre obtained in some readings is probably a slight variation of the zero, and is much too small in value to be regarded as a true pressure.

2. The experiment was commenced with a small positive pressure artificially produced.

only the fluid portion was taken for determination of percentage of starch. The determination was carried out as before by drying to a constant weight, and gave 1.82 per cent. of starch.

*Experiment IV.*—This experiment was continued for eleven days, and showed zero osmotic pressure like all the preceding ones. Total percentage of starch, 6.02.

#### EXPERIMENTS ON THE HYDROLYTIC PRODUCTS FROM STARCH.

In these experiments, ptyalin was allowed to act upon starch for the varying intervals mentioned in each case. The results show that at a certain stage at which the blue reaction has almost disappeared a non-diffusible dextrin is present, giving a constant osmotic pressure, but further action makes the whole diffusible, and no constant osmotic pressure is obtained. It may also be noted that erythrodextrin, or some substance giving a red colouration with iodine, diffuses through the parchment paper.

*Experiment I.*—A starch mucilage acted upon by saliva (1 c.c. in 100 c.c.) at 40° C. for ten seconds, then heated to 75° C. and filtered. The mucilage became quite fluid in the process, showing that 'soluble starch' had been formed. No osmotic pressure was obtained throughout a period of eight days. The solution taken out at the end was more opalescent than at the beginning. The water side when tested gave a red colour with iodine and reduced Fehling's solution. It contained 0.09 per cent. of dried solids. The starch side contained 3.44 per cent. of dried solids, and 0.0012 per cent. of ash.

*Experiment II.*—In this experiment saliva was added, 1 c.c. in 100 of starch mucilage, and without destroying the ptyalin this was placed in the osmometer. No osmotic pressure was obtained throughout a period of eight days.

*Experiment III.*—One hundred c.c. of starch mucilage acted upon by 1 c.c. of saliva for 17 minutes at 40° C.; blue colour gone, red of erythrodextrin present. Ptyalin destroyed by boiling; filtered, and put into osmometer.

	Time from commencement		Osmotic Pressure in mm. of mercury	Temperature in degrees Centigrade		
	Days	Hours				
October 24th, 1904 ...	0	0	...	0	...	15 <sup>c</sup>
	0	9	...	2	...	16 <sup>c</sup>
	1	0	...	18	...	13 <sup>o</sup>
	2	0	...	16	...	14 <sup>o</sup>
	3	6	...	26	...	14 <sup>o</sup>
	4	0	...	26	...	13 <sup>o</sup>
	5	0	...	26	...	13 <sup>c</sup>



There is accordingly in this stage a definite and steady pressure. The solution side contained 2.37 per cent. of dried solid at the end, about half of which was precipitable by alcohol; the water side contained 1.11 per cent. of total solid, of which only a trace was precipitable by alcohol.

#### EXPERIMENTS WITH SUGARS.

The experiments with the products of hydrolysis of starch, show that the sugars pass easily through parchment paper, and give no osmotic pressure effect in the osmometer. The matter was, in addition, tested directly by two experiments, one on maltose and the other on arabinose.

The experiments are of interest in showing how completely equality of concentration is attained on the two sides of the membrane, and suggested a method for the quantitative determination of sugar in blood and its derived fluids, which possesses many practical advantages.

*Experiment I.*—A solution of maltose made up in cold water was placed in osmometer, against distilled water on the other side. The experiment was allowed to run for six days, and no trace of osmotic pressure was obtained throughout, and on opening the clip at the end of the experiment not the slightest movement was seen in the mercury of the manometer. Analysis showed the same amount of maltose on the two sides within the limits of experimental error, viz., 0.35 per cent. on water side, and 0.38 on solution side.

*Experiment II.*—A similar experiment with arabinose; experiment kept up for five days; no pressure shown throughout; analysis showed 0.35 per cent. on water side, and 0.36 per cent. on solution side.

#### EXPERIMENTS WITH GUMS

*Gum Acacia.*—*Experiment I.*—A 6 per cent. solution of specially hand picked gum acacia was made up and placed in osmometer against distilled water. The experiment was continued for six days, for the last two of which the pressure was steady.

	Time from commencement		Osmotic Pressure in mm. of mercury	Temperature in degrees Centigrade
	Days	Hours		
September 15th, 1904, 9 a.m.	0	0	...	15
	1	0	...	15
	1	2	...	16
	2	0	...	17
	4	0	...	16
	5	0	...	16
	6	0	...	15

Analyses of the two fluids at the end showed 0.006 per cent. of total solids on the water side, and 6.25 per cent. of total solids on the solution side of which 6.09 per cent. was organic, and 0.16 per cent. was inorganic ash.

In two other experiments similar results were obtained, a 6 per cent. solution giving an osmotic pressure of 142 mm. at 15° C., and a 10 per cent. solution an osmotic pressure of 276 at 13° C.

*Gum Tragacanth.*—This gum while it swells up enormously in water does not dissolve readily even in hot water, and the small amount used (0.72 per cent.), which is, however, sufficient to form a thick mucilage, was the highest concentration with which we were able to experiment. This solution showed a small pressure of 5 mm. of mercury at 17° C. The pressure was raised artificially to 20 mm., but slowly fell again. This shows that if gum tragacanth possesses an osmotic pressure at all in water it is scarcely measurable even by the direct method.

### URIC ACID SOLUTION.

In certain respects, such as precipitation in gelatinous forms from solution, and salting out with crystalloids, uric acid and certain urates appear to behave as colloids. It was hence thought of interest to determine whether a saturated solution of uric acid in distilled water exhibited any osmotic pressure, and whether it passed through a parchment paper membrane.

The saturated solution was prepared by boiling up excess of uric acid with distilled water, and filtering hot from the chief excess, and again from re-precipitated uric acid after cooling.

The result showed no appearance of osmotic pressure throughout an experiment lasting for five days (at 15° to 16° C.), and at the end of that period, practically the same concentration in uric acid on the two sides of the membrane.

The determinations were made by titration with potassium permanganate in the usual way, with the following results:—

Ten c.c. of original solution placed in osmometer require 0.5 c.c. of  $\text{KMnO}_4$  solution = 1.875 in 10,000.

Ten c.c. on uric acid side at end require 0.25 c.c. = 0.9375 in 10,000.

Ten c.c. on water side at end require 0.22 c.c. = 0.8250 in 10,000.

This shows that uric acid behaves in solution in water as a crystalloid, and readily diffuses through the membrane.

EXPERIMENTS ON BLOOD SERUM AND ITS CONSTITUENTS AND  
EFFECTS OF REAGENTS.

*Experiment 1.*—In this experiment it was sought to determine whether the presence of anaesthetics or organic solvents altered the osmotic pressure of the serum proteids. No change in osmotic pressure was obtained, and the experiment shows how closely readings come to the same point with the same sample of serum. Also, since the chloroform used acted as a preservative from any chance of bacterial growth, the results show that the osmotic pressure readings obtained were not due to any crystalloidal products of proteid decomposition. For brevity sake, only one daily reading is given, and the intermediate readings, which were consistent with these, are omitted.

The experiments were carried out in July, 1904.

No. 1.—*Serum (pig's) only, against 0.75 per cent. saline.*

Time d. h.		Osmotic Pressure mm.		Temperature	
0	0	...	0	...	17° C.
1	0	...	12	...	17°
2	0	...	24	...	18°
3	0	...	32	...	20°
4	0	...	30	...	20°
5	5	...	31	...	19°
5	22	...	30.5	...	18°

No. 2.—*The same serum saturated with chloroform, against 'saline (0.75 per cent.) also saturated with chloroform.*

Time d. h.		Osmotic Pressure mm.		Temperature	
0	0	...	0	...	19° C.
0.	21	...	22	...	18°
1	5	...	28	...	18°
1	21	...	31	...	18°
2	0	...	31	...	18°

No. 3.—*The same serum saturated with benzol, against 0.75 per cent. saline, also saturated with benzol.*

Time d. h.		Osmotic Pressure mm.		Temperature	
0	0	...	0	...	19° C.
1	0	...	34	...	18°
2	0	...	34	...	19°
2	19	...	34	...	19°

These experiments show that there is no appreciable change in the solution aggregate caused by the anaesthetic. It may be pointed

out that the amount of chloroform on the serum side, as shown by Moore and Roaf's<sup>1</sup> experiments, would, at the saturation point, be four or five times as great as on the saline side, and since this does not appreciably disturb the osmotic pressure, it must be in combination with the proteid.

*Experiment II.*—This experiment was on the effects of converting the serum proteids into alkali albumen by boiling with 1 per cent. caustic potash, and measuring osmotic pressure against saline (0.75 per cent.) to which an equal amount of caustic potash had been added. The result showed an immense rise in osmotic pressure, similar to that obtained by Moore and Parker (*loc. cit.*).

The experiments were carried out in December, 1904, and are at a lower temperature than the preceding one.

No. 1.—Serum, untreated, against saline (0.75 per cent.)

Time		Osmotic Pressure		Temperature
d.	h.	mm.		
0	0	...	0	12° C.
1	2	...	10	12°
2	0	...	14	12°
8	16	...	18	12°

Percentage of proteid = 7.97

No. 2. The same serum boiled with 1 per cent. of potassium hydrate against saline (0.75 per cent.) plus 1 per cent. of potassium hydrate.

Time			Osmotic Pressure		Temperature
d.	h.		mm.		
0	0	...	0	...	9° C.
0	5	...	12	...	10°
0	7	...	24	...	11°
0	11	...	40	...	11°
1	0	...	80	...	9°
1	9	...	94	...	11°
2	0	...	100	...	10°
6	0	...	103	...	7°
7	0	...	103	...	8°

Percentage of proteid 5.02.

The action of the caustic potash causes the osmotic pressure to rise to between five and six times its former value, and the increased pressure remains steady.

1. *Proc. Roy. Soc.*, Vol. LXXIII, 1904, p. 382. *Ibid.* B. Vol. LXXVII, 1905, p. 86.  
Thompson Yates & Johnston Lab. Reports, Vol. VI, Pt. I, 1905, p. 151.



*Experiment II.*—The same result was obtained in this experiment in which the globulin and albumin were separated, and their osmotic pressures taken before and after treatment by boiling with 1 per cent. of potassium hydrate. In the latter case, 1 per cent. of potassium hydrate was also added to the 0.75 per cent. saline on the other side of the membrane, as in the preceding experiment.

The globulin was obtained by half saturation with ammonium sulphate, and was twice washed and then made up to the volume of the original serum; the serum albumin was twice precipitated by saturation with ammonium sulphate, and then dialysed and made up to the volume of the original serum. The alkali-globulin or -albumin was made by adding 1 per cent. of potassium hydrate and raising the temperature to the boiling point.

The osmotic pressure rose, as in the previous experiments, to a constant maximum. To save space, only this maximum is given in each case.

Substance	Osmotic pressure	Temperature
Serum ... ..	18 mm.	12° C
Alkalized serum ... ..	103 „	8°
Serum-globulin ... ..	7 „	13°
Serum albumin ... ..	4 „	12°
∴ Serum albumin + serum globulin ... ..	11 „	—
Alkalized serum globulin	62 „	10°
Alkalized serum albumin	44 „	12°
∴ Alkalized serum-globulin + alkalized serum- albumin ... ..	106 „	—

It is evident from these figures that the precipitation and washing of the serum proteids changes these so that the sum of the pressures is less than that of the total proteids of the serum in their natural condition; but that alkalization leads to osmotic pressures which give for their sum a pressure practically the same as that obtained by alkalization of the serum directly.

In all cases, the biuret test gave, at the end, a negative result in the fluids on the water side.

*Experiment III.*—On the effects of addition of considerable amounts of neutral salt on the osmotic pressure of the serum proteids. Since a large amount of neutral salt added to a proteid-containing solution has the effect of salting out the proteid in many cases, the present experiment was devised to determine whether, at a point short of precipitation, any increased aggregation occurred as shown by diminished osmotic pressure. Two experiments were carried out, in each of which a control of the same

serum, against 0.9 per cent. sodium chloride solution, was used. The second osmometer had 20 per cent. of crystallised magnesium sulphate ( $\text{MgSO}_4 + 5 \text{H}_2\text{O}$ ) added on each side of the membrane, so that on one side there was serum + 20 per cent. crystallized magnesium sulphate, and on the other, 0.9 per cent. sodium chloride plus 20 per cent. magnesium sulphate. Since the two experiments gave similar results, details are only given in the case of one.

## No. 1

Pig's serum against 0.9 per cent. saline.

## No. 2

Pig's serum + 20 per cent. of cryst. mag. sulph. against 0.9 per cent. saline + 20 per cent. cryst. mag. sulph. after dialysing the two fluids against each other.

Time from commencement d. h.		Osmotic pressure in mm. of mercury		Time from commencement d. h.		Osmotic pressure in mm. of mercury	
0	0	...	0	...	0	0	...
1	3	...	14	...	1	2	...
2	3	...	21	...	2	2	...
2	18	...	24	...	2	17	...
3	18	...	26	...	3	17	...
4	18	...	27	...	4	17	...
5	20	...	27	...	5	19	...
6	18	...	27	...	6	17	...
7	18	...	27	...	7	17	...
8	18	...	27	...	8	17	...
10	18	...	27	...	10	17	...
12	19	...	27	...	12	18	...
14	18	...	27	...	14	17	...
16	18	...	27	...	16	17	...

The figures show that the maximum pressure in the salted serum rose to 31 mm. at the same period as the untreated serum stood at 27 mm.; that later, the pressure in the salted serum fell to 18 mm., while that in the unsalted remained steadily at 27 mm.

Analyses at the end showed, on the serum side, 85.62 per cent. of water, 5.93 per cent. of organic matter, and 8.45 per cent. of  $\text{MgSO}_4$ ; on the saline side, 90.82 per cent. of water, and 9.18 per cent. of  $\text{MgSO}_4$ , thus, the amount of saline is closely proportional to the percentage of water on each side.

The second experiment showed, in the salted serum, a maximum pressure of 32 mm. in two days, which fell to a constant 18 mm. in eight days; while the untreated serum rose to a maximum of 21 mm., and remained constant at that level until the experiment was terminated in ten days.

In our opinion, these changes are not due to diffusion of magnesium sulphate, for diffusion in our osmometer of such easily soluble crystalloids occurs very rapidly, while these changes in osmotic pressure occupied several days. They seem, therefore, to be due to some slow molecular or aggregation change induced by the excess of salt.

#### EXPERIMENTS WITH LECITHIN AND LANOLINE MEMBRANES.

Since it has been supposed that the peculiarities in the behaviour of cells to certain electrolytes and inorganic ions are due to a cell membrane which is permeable to some and impermeable to others, and that this membrane is composed of, or consists chiefly of, lecithins and other lipoids, which confer upon it these peculiar properties of selective permeability and impermeability, it was considered of some importance to prepare such membranes in the pores of parchment paper and coating its surfaces, and to test whether such a membrane showed similar permeabilities to those *supposed* by many authors to be shown by cells, and, if so, whether osmotic pressures would be developed when ions to which the membrane was not permeable were present in different concentrations on the two sides.

Both lecithin and lanoline membranes were prepared and tested, and it was found that these were readily permeable and that no trace of osmotic pressure was developed.

This demonstrates, in the first place, that a lipoid membrane does not furnish any explanation of the osmotic properties of living cells, and, secondly, that the presence or absence of an inorganic ion in a cell, or variations in concentration of such an ion inside and outside of the cell, are not to be ascribed to a barrier opposed by such a membrane, but that the explanation is rather to be sought in the

properties of the cell contents themselves, for combining with or adsorbing such constituents of its inorganic environment.

The lecithin was prepared by the method described by Roaf and Edie,<sup>1</sup> and dissolved in the smallest portion possible of ether. The discs of parchment paper, cut to the proper size to fit the osmometer, dried at 100° C. and cooled in a desiccator, were dipped in this strong lecithin solution and the ether allowed to evaporate off; they were then dipped a second time and allowed to dry again. In this manner, the parchment paper is thoroughly soaked with lecithin, becomes translucent, and is coated with a thin layer of lecithin on both surfaces.

*Experiment I.*—A membrane, impregnated with lecithin as above described, was fitted into the osmometer, which was filled with 1 per cent. sodium chloride solution on one side, and with 0.5 per cent. sodium chloride solution on the other. This arrangement was used in preference to distilled water on one side, in order to keep the lecithin from emulsifying in the water. The experiment was kept going for five days, during which time the pressure remained at zero, and afterwards a small positive pressure produced artificially slowly disappeared. The fluids on the two sides were titrated for chlorides, and the originally 1 per cent. solution was now found to be 0.89 per cent., and the originally 0.5 per cent. was now 0.52. From these figures it appears as if some of the salt had been adsorbed by the lecithin, but, in addition, the absence of pressure throughout, and the increase on the more dilute side, show that the membrane is permeable to chlorides. Also, the entire absence of pressure, in face of such a difference in concentration, clearly shows that the osmotic phenomena of the living cell, and the wide differences in the qualitative composition of the inorganic salts of cell and of plasma or lymph, cannot be due to the presence, surrounding the cell, of an impermeable membrane of lecithin which prevents ingress or egress of certain inorganic ions.

There is no experimental evidence of the existence of any impermeable membrane surrounding cells, and the differences of composition within and without are, in our opinion, more probably due to the special affinities of the cell protoplasm for different ions than to the action of any such hypothetical membrane.

*Experiment II.*—A similar experiment, but with a membrane of lanoline instead of lecithin, gave similar results—no osmotic pressure being obtained, and a rise in concentration in chlorides on the less concentrated, and a fall on the more concentrated side.

<sup>1</sup> Thompson Yates & Johnston Laboratories Reports, Vol. VI, Pt. I, 1905, p. 201.



## CONCLUSIONS

1. Evidence is given by direct measurement that certain colloids, which cannot be shown to definitely possess osmotic properties by the indirect methods, such as depression of freezing point or raising of boiling point, do show a very definite and easily measurable osmotic pressure when a membrane permeable to crystalloids is used in the osmometer.

2. As evidence that the pressure so obtained is due to colloid, and not to crystalloid, the following facts are brought forward :—

(a) Colloids diffuse in solution and hence must have a falling gradient of osmotic potential.

(b) The pressure reaches a maximum, and there remains constant ; if it were due to crystalloid not attached to colloid, this pressure would rise and then fall gradually to zero.

(c) The pressure is modified by varying amounts of easily diffusible crystalloids in solution, provided such crystalloids affect the condition of the colloids, although such crystalloids pass with ease and rapidity through the membrane, and accordingly themselves show no permanent osmotic pressure.

(d) Anything which is capable of altering the condition of the colloid, such as alkalization of the serum proteids, or hydrolysis of starch, causes corresponding changes in the osmotic pressure.

(e) Repeated precipitation of an alterable colloid, such as the serum proteids, causes a fall in osmotic pressure ; but this effect subsequently disappears on alkalization, the pressure for the fractions of alkalized proteid adding up to the total for the fresh serum alkalized as a whole.

3. Certain colloids, such as starch (potato) and probably gum tragacanth, have so high a state of aggregation that they show no osmotic pressure, even by the direct measurement ; those showing osmotic pressure clearly, which have been dealt with in this paper, are the serum proteids, gelatine and gum acacia.

4. At the stage of hydrolysis of starch at which the blue colour with iodine has just disappeared, the dextrans present give a permanent osmotic pressure.

5. In the case of gelatine the effect of variation of temperature upon the osmotic pressure has been observed. It has been found that the osmotic pressure rises with the temperature, and that the rise is more than proportional to the absolute temperature, indicating a dissociation and lowering of the solution aggregate as the temperature is raised. Prolonged heating to 80° C. or over causes a permanent change with higher osmotic pressure; but heating for a short period gives a higher pressure, which persists for a few days, after which the osmotic pressure returns to its former value, and the physical properties of the gelatine solution are not permanently altered.

6. All the sugars experimented with behave as true crystalloids in passing readily through and equalizing on the two sides, and hence show no osmotic pressure; uric acid solutions also behave as true crystalloids.

7. Serum globulin and serum albumin both possess osmotic pressure, but the process of separation has the effect of altering the state of aggregation so that the sum of the pressure shown by the two fractions is less than that of the serum from which they came; but on alkalization the sum of the pressures equals that of the alkalized serum.

8. Addition of a considerable quantity of a neutral saline (magnesium sulphate) to serum, causes, initially, a rise in osmotic pressure, followed by a fall to a constant lower level, which is less than that given by the untreated serum.

9. The slow change in osmotic pressure observed in colloidal solutions, such as that back to normal conditions in gelatine after a short period at a higher temperature, and of the fall observed after addition of magnesium sulphate to serum, which requires some days to be completed, indicates a kind of hysteresis in such solutions, or a very slow return to equilibrium after the state of aggregation has been disturbed.

10. A lecithin or lanoline membrane is not impermeable to crystalloids, and hence shows no osmotic pressure when the concentration of crystalloids is different on its two sides.

Accordingly, the supposed presence of such a membrane will not explain the peculiar content of the cell in crystalloids, different from that of the bathing lymph around it. A more probable view is that the cell protoplasm has selective adsorptive powers for different ions, and that such ions exist in the cell in combination or adsorption with the cell substance.

EVIDENCE OF THE EXISTENCE OF A PRE-OPSONIN  
IN NORMAL SERUM, CONVERTIBLE INTO ACTIVE  
OPSONIN BY THE ADDITION OF ANY MICRO-  
ORGANISM — LOWERING AND ULTIMATE SUP-  
PRESSION OF OPSONIC POWER OF NORMAL SERUM  
TO ALL ORGANISMS BY THE ADDITION OF ONE.

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It is now a well-known fact that the sera of the higher animals contain opsonin for many different bacteria. Thus, staphylococci and streptococci and tubercle, typhoid, colon, anthrax, tetanus, diphtheria, and many other bacilli, when treated with normal human serum, are rendered susceptible to phagocytosis by leucocytes.

One of the questions which a knowledge of this fact immediately suggests is, whether the action of the serum is due to one substance in it capable of acting on almost any micro-organism with which it may come in contact, or whether it is the result of an enormous number of distinct bodies, each of which opsonises only one particular kind of bacteria or group of bacteria. In other words, is the generally accepted view that there exist in normal serum specific opsonins for each different micro-organism correct: or is there a single body, pre-opsonin, which, under the stimulus of the presence of a micro-organism, produces an active opsonin for that organism?

The current view, that in normal serum a high degree of specificity exists, depends upon evidence of the following nature.

In the first place, an individual may have a high opsonic index for one organism and be normal to all others, or he may be low to one bacterium and normal to the remainder. For example, the inoculation of a normal man with tuberculin will cause a rise or fall in his tuberculo-opsonic index, without any alteration in his opsonic



power for other organisms. Again, persons suffering from some such disease as acne or chronic tuberculosis often have an abnormal opsonic index for either staphylococcus or tubercle, whereas their other indices are normal.

In the second place, it has been noticed that the addition of tubercle bacilli to normal human serum, and incubation of the mixture at 37° C. for a period, will deprive the serum, when separated from the bacilli by prolonged centrifugalization, of its tuberculo-opsonin, whereas the opsonin for staphylococci remains unaffected; and conversely, serum treated in a similar manner with staphylococci, loses its staphylococcic opsonin, but retains its tuberculo-opsonin.<sup>1</sup>

From observations of this kind it has been argued that, if the serum of a tubercular patient can have a high tuberculo-opsonic index and a normal one for anthrax or typhoid bacilli, and if the addition of staphylococci to serum reduces its opsonic index for this organism but not for tubercle bacilli, then there must be present in serum a large number of specific opsonins.

On reflection, however, it seems hardly probable that normal serum contains an enormous number of different opsonins, all more or less distinct from one another, each present only for the purpose of defending the body from its corresponding bacterium, which it is quite probable it may never be required to do.

With a view to investigating this point more fully we performed a number of experiments of the following nature. Various amounts of different micro-organisms were suspended in separate portions of normal human serum, and after longer or shorter periods of incubation were thrown down by prolonged centrifugalization. The opsonic indices of the supernatant serum for different bacteria were then estimated.

#### EXPERIMENTAL RESULTS

The following are some of the results :—

*Experiment I.*—Two portions of the same normal human serum were taken, A and B. Living anthrax bacilli were added to A, and both A and B were then placed in the

1. Bulloch and Western. *The Lancet*, 1905, Vol. II, p. 1603.

incubator at 37° C. for 15 minutes. The bacteria were then thrown down as a deposit by prolonged centrifugalization. The opsonic index of the supernatant serum A was then determined for anthrax bacilli and for staphylococci, the other portion, B, being used as a control.

			No. of bacteria phagocytosed		No. of leucocytes counted		Index
ANTHRAX—							
Control serum	...	...	67	...	50	...	1
Treated serum	...	...	19	...	50	...	3
STAPHYLOCOCCUS—							
Control serum	...	...	210	...	50	...	1
Treated serum	...	...	91	...	50	...	4

*Experiment II.*—The period of incubation was prolonged to 45 minutes.

			No. of bacteria phagocytosed		No. of leucocytes counted		Index
ANTHRAX—							
Control serum	...	...	59	...	50	...	1
Treated serum	...	...	17	...	50	...	28
STAPHYLOCOCCUS—							
Control serum	...	...	154	...	50	...	1
Treated serum	...	...	23	...	50	...	15

*Experiment III.*—In this experiment the sera were allowed to stand for two hours at the temperature of the room instead of being placed in the incubator.

			No. of bacteria phagocytosed		No. of leucocytes counted		Index
ANTHRAX—							
Normal serum	...	...	89	...	50	...	1
Treated serum	...	...	4	...	50	...	0
STAPHYLOCOCCUS—							
Normal serum	...	...	220	...	50	...	1
Treated serum	...	...	46	...	50	...	2

*Experiment IV.*—In this experiment staphylococci were added to the serum instead of anthrax, and the mixture was incubated 45 minutes.

			No. of bacteria phagocytosed		No. of leucocytes counted		Index
ANTHRAX—							
Normal serum	...	...	121	...	50	...	1
Treated serum	...	...	9	...	50	...	0
STAPHYLOCOCCUS—							
Normal serum	...	...	430	...	50	...	1
Treated serum	...	...	9	...	50	...	0

*Experiment V.*—Here anthrax bacilli, killed by heating at 100° C. for 15 minutes and then, after cooling, heated again at 100° C. for 15 minutes, were added to serum, and the experiment continued as in I.

A When incubated with dead anthrax for 15 minutes.

			No. of bacteria phagocytosed	No. of leucocytes counted			Index
ANTHRAX—							
Control serum	...	...	45	...	40	...	1
Treated serum	...	...	1	...	40	...	10
STAPHYLOCOCCUS—							
Control serum	...	...	118	...	40	...	1
Treated serum	...	...	36	...	40	...	13

B When incubated with dead anthrax for 45 minutes.

			No. of bacteria phagocytosed	No. of leucocytes counted			Index
ANTHRAX—							
Control serum	...	...	45	...	40	...	1
Treated serum	...	...	0	...	40	...	10
STAPHYLOCOCCUS—							
Control serum	...	...	118	...	40	...	1
Treated serum	...	...	6	...	40	...	10

*Experiment VI.*—Dead tubercle bacilli were added to serum, and the mixture incubated for 15 minutes. Then, after separating the micro-organisms by means of the centrifuge, the opsonic power of the supernatant serum was estimated for tubercle and staphylococci.

			No. of bacteria phagocytosed	No. of leucocytes counted			Index
TUBERCLE—							
Control serum	...	...	239	...	50	...	1
Treated serum	...	...	49	...	50	...	12
STAPHYLOCOCCUS—							
Control serum	...	...	376	...	50	...	1
Treated serum	...	...	140	...	50	...	137

In all the preceding observations there was no definite relation between the amount of serum used and the quantity of bacteria added. It varied in each experiment, a quantity of the bacteria obtained from a growth on agar being used in each case, except Experiment VI.

*Experiment VII.*—A strong emulsion of anthrax bacilli was made in 9 per cent. NaCl. This was heated at 100° C. for 30 minutes. The bacilli were then thrown down

as a deposit by means of the centrifuge, and washed with '9 per cent. NaCl. This was repeated three times. These washed dead bacilli were then made up into a strong emulsion in '9 per cent. NaCl., and added to three separate quantities of normal serum in the following proportions—

- A 1 part of emulsion to 19 parts of serum.
- B 5 parts of emulsion to 15 parts of serum.
- C 10 parts of emulsion to 10 parts of serum.

These were then incubated at 37° C. for 15 minutes, and after centrifugalization, the opsonic index of the supernatant sera for anthrax and staphylococci was determined as before. In each case the control sera were diluted equally with '9 per cent. NaCl.

			No. of bacteria phagocytosed		No. of leucocytes counted		Index
A ANTHRAX—							
Control serum	...	...	222	...	50	...	1
Treated serum	...	...	170	...	50	...	'7
STAPHYLOCOCCUS—							
Control serum	...	...	382	...	50	...	1
Treated serum	...	...	227	...	50	...	'6
B ANTHRAX—							
Control serum	...	...	173	...	50	...	1
Treated serum	...	...	58	...	50	...	'3
STAPHYLOCOCCUS—							
Control serum	...	...	433	...	50	...	1
Treated serum	...	...	179	...	50	...	'4
C ANTHRAX—							
Control serum	...	...	135	...	50	...	1
Treated serum	...	...	14	...	50	...	'1
STAPHYLOCOCCUS—							
Control serum	...	...	369	...	50	...	1
Treated serum	...	...	165	...	50	...	'4

*Experiment VIII.*—A strong emulsion of washed dead anthrax bacilli was prepared as in Experiment VII. Equal quantities of this were added to two equal portions of normal serum, A and B.

A was incubated for 30 minutes at 37° C.

B was incubated for 60 minutes at 37° C.

Then, after throwing down the bacteria by means of the centrifuge, the supernatant sera were tested for opsonins as before with staphylococci and anthrax. The control sera were diluted to a similar extent with '9 per cent. NaCl, and were placed in the incubator for corresponding periods.



		No. of bacteria phagocytosed		No. of leucocytes counted		Index
A	ANTHRAX—					
	Control serum	...	40	...	50	1
	Treated serum	...	8	...	50	2
	STAPHYLOCOCCUS—					
	Control serum	...	341	...	50	1
	Treated serum	...	87	...	50	25
B	ANTHRAX—					
	Control serum	...	48	...	50	1
	Treated serum	...	2	...	50	10
	STAPHYLOCOCCUS—					
	Control serum	...	395	...	50	1
	Treated serum	...	70	...	50	17

From these experiments it would seem that it is impossible to separate from normal serum any one opsonin by adding the particular microbe to it, and at the same time leave the opsonins corresponding to other micro-organisms intact. It appears that adding a quantity of any microbe to serum, will enormously diminish its opsonic power for other organisms, as well as for that corresponding to the bacteria added. The fall in the opsonic index is, apparently, general, in contradiction to what one would expect if there were a number of distinct opsonins in the serum.

These facts are more easily explicable on the supposition that there is in normal serum a single body from which, under the adequate stimulation of certain bacteria, there can be split off the particular opsonin which will combine with them.

Normal serum will no longer exert an opsonic effect upon bacteria, as measured by Wright's method, after it has been heated at 60° C. for 15 minutes. Immune opsonins, on the contrary, are but little affected by such a temperature.

It has been shown that cocci, which have been suspended in normal serum for some time, are, even after heating at 60° C. for several hours, readily ingested by leucocytes.<sup>1</sup>

From these observations it may be argued that there is present in normal serum a substance '*Pre-opsonin*.' This, when it comes in contact with any kind of bacteria splits off from itself the specific

1. Bulloch and Atkin, *Proc. Roy. Soc.*, Vol. LXXIV, p. 384.

opsonin necessary for the bacterium. The greater the number of any kind of bacteria added to any portion of serum, the more specific opsonin it is necessary for the pre-opsonin to form. This must necessarily mean a lessening in the quantity of pre-opsonin present. Hence, less quantities of other specific opsonins are formed when other organisms are added to the serum. This will account for the general lowering of the opsonic indices which we noted in the foregoing experiments.

The specific opsonin which becomes attached to the bacteria may be the same thing as the immune opsonin, since, as we have seen, both are unaffected by heating to 60° C.

The specific rise in the opsonic index which is noticed after the injection of a vaccine may be explained by assuming that certain of the opsonin-forming cells are stimulated to form a specific opsonin instead of, or as well as, the general pre-opsonin which they are normally putting out into the serum.

We have noticed that after the injection of a healthy man with anti-tetanic serum a specific rise in the tetano-opsonic index was followed by a general fall. Thus the staphylo-, tuberculo-, and tetano-opsonic indices, all fell considerably below normal. A similar, though less marked, general depression was observed after injection of anti-streptococcic and anti-diphtheritic sera<sup>1</sup>. The tuberculo-opsonic indices of nine patients inoculated with anti-diphtheritic serum, were all found to be low.<sup>2</sup>

It is possible that these anti-toxic and anti-bacterial sera resulted in the formation of specific anti-opsonins.

Hektoen and Ruediger mention a number of non-specific anti-opsonins, e.g.,  $\text{CaCl}_2$ ,  $\text{BaCl}_2$ ,  $\text{NaHCO}_3$ , lactic acid, alcohol, etc.<sup>3</sup>

That these specific anti-opsonins and non-specific anti-opsonins result in a general fall in the opsonic action of the blood, seems to favour the view that, in normal serum, there are probably not a considerable number of distinct opsonins.

1. Yorke and Smith, *Bio-Chemical Journal*, Vol. I, 1906. p. 341.

2. Bradshaw, *The Lancet*, 1906, May 19.

3. Hektoen and Ruediger, *Journal of the American Med. Assoc.*, May, 1906.

# ON EXTRACTION BY CASEIN OF TRYPSIN, ADSORBED BY CHARCOAL

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*(Received December 3rd, 1906)*

According to recently published investigations of mine, charcoal exercises an anti-tryptic action, which can be divided up into two consecutive stages, namely, (1) the taking up or adsorption of the trypsin by the charcoal, and (2) the fixation of the trypsin.<sup>1</sup> The mere adsorption could, in absence of proteins, very readily be made complete, as shown by the fact that, after filtering off the charcoal, the filtrate showed no tryptic activity. On the other hand the fixation was never found to be complete as borne out by the observation that a mixture of charcoal and trypsin, even after complete adsorption, gave some tryptic effect with added casein. This was found to depend upon the casein extracting part of the enzyme from the charcoal. The tryptic effect obtained after complete adsorption therefore represents in a way the amount of trypsin adsorbed but not fixed. Furthermore it could be shown that, after proper treatment with casein solution and filtering off the charcoal, the filtrate gave the same tryptic effect as the non-filtered charcoal-trypsin-casein mixture. This proves that all the trypsin that is able to act upon the casein can be made to pass through the filter together with the casein—pointing to some kind of combination being formed between casein and the trypsin. This is in full agreement with the view generally accepted, according to which the action of enzymes is made up of two consecutive stages—(1) combination of the enzyme with the substrate, and (2) breaking up of the latter.

1. *Bio-Chemical Journal*, I, p. 484, 1906.

Up to the present time it has not been possible, experimentally, to distinguish between these two stages, or to study the one independently of the other. The experiments referred to open, I think, a way to studying the process of combination by itself.

If a mixture of a sufficient amount of charcoal and trypsin (approximately free from proteins) is kept for twenty-four to forty-eight hours, then the adsorption will be completed, and the fixation of the trypsin to the charcoal will be so nearly finished that the amount of trypsin adsorbed but not fixed can be considered as constant, anyhow during a short interval of time (one to two hours). By means of added casein-solution, part of the trypsin can be extracted from the charcoal and attached to the casein, and this process can be interrupted at any time by filtering off the charcoal. The tryptic effect obtained with the non-filtered charcoal-trypsin-casein mixture is produced by the whole amount of trypsin transferable under the conditions observed, and the digestive power of the filtrate is produced by the amount of trypsin combined with the casein before the filtration took place. The process of extraction is finished when filtered and non-filtered specimens give the same effect; the extraction is likewise finished when two samples, obtained by filtering after different intervals of time, show the same digestive power.

In the experiments of which I am going to give an account, the influence of the following factors upon the amount of trypsin extracted by casein from charcoal has been tried :—

1. The temperature.
2. The time of interaction.
3. Amount of water present.
4. Amount of casein.

In all the specimens digested toluol was used as antiseptic, and the effect of the trypsin extracted was determined, as described in previous papers of mine, by estimating the amount of nitrogen in equal volumes of the tannic acid filtrates.<sup>1</sup>

1. *Journ. of Physiol.*, XXXII, p. 390, 1905.



All my experiments were carried out with charcoal-trypsin mixtures in which complete adsorption had been effected. Therefore, all the tryptic effect obtained was due to trypsin which had been transferred from the charcoal to the casein used. According to the investigations referred to, the amount of trypsin transferable is less the larger the amount of charcoal used, the longer the time of interaction between the charcoal and the trypsin before the casein is added, and the higher the temperature for this interaction.<sup>1</sup> Since the trypsin used was not the same in all experiments, and other conditions likewise were varied, only the figures obtained in the same experiment are comparable.

#### INFLUENCE OF THE TEMPERATURE

*Experiment I.*—The charcoal-trypsin mixture had been kept for twenty-four hours at 37°. Equal volumes of this charcoal-trypsin and of casein solution<sup>2</sup> were mixed and kept at 20° and at 0°, and after the intervals of time recorded the resulting mixtures were filtered at the same temperatures. Then equal volumes of the filtrates were *digested at 37°* for two days. At the same time one specimen which had not been filtered was digested. This specimen, therefore, showed a digestive power corresponding to the amount of trypsin transferable at 37°, and the filtered samples gave the amounts transferred at 20° and at 0°.

The results were:—

		37°		20°		0°
Unfiltered specimen	...	9.7	...	—	...	—
Filtered after $\frac{1}{2}$ hour	...	—	...	4.4	...	2.4
“ “ I “	...	—	...	4.6	...	2.45
“ “ $1\frac{1}{2}$ “	...	—	...	4.45	...	—

Therefore the extraction was finished after half an hour, both at 20° and at 0°, and the experiment shows that *more trypsin is transferred the higher the temperature.*

*Experiment II.*—The charcoal-trypsin was kept at 20° for two days. Then equal charcoal-trypsin-casein mixtures were prepared, kept and filtered at 37° and at 18°. Equal volumes of the filtrates and one unfiltered specimen, were all *digested at 37°* for two days.

		37°		18°
Filtered after $\frac{1}{2}$ hour	...	5.85	...	5.3
“ “ I “	...	6.4	...	5.25
Unfiltered specimen	...	8.1	...	—

1. *Bio-Chemical Journal*, I, p. 495, 1906.

2. The solutions used contained 2.5 per cent. of casein in 0.2 per cent.  $\text{Na}_2\text{CO}_3$  solution.

At 18° the extraction was completed after half an hour, but at 37° it was not finished in one hour, since the total amount of trypsin transferable at 37° is represented by the figure 8·1, obtained from the-unfiltered specimen. The experiment confirms the result of Experiment I as to the influence of the temperature upon the amount of trypsin extracted, and in addition it shows that the extraction comes to an end more speedily at 18° than at 37°.

### INFLUENCE OF THE TIME OF INTERACTION

*Experiment III.*—The charcoal-trypsin was kept at 20° for twenty-four hours. Then mixtures were prepared and kept at 20° as follows:—

30 c.c. charcoal-trypsin +	150 c.c. cas. sol.	...	...	A
„ „ +	15 c.c. „	...	...	B

After the intervals of time recorded, specimens of the mixture were filtered. Of A 30 c.c. filtrate was digested by itself, and of B 7·5 c.c. filtrate was digested together with 22·5 c.c. casein solution. All specimens were digested at 37° for twenty-four hours, and the effect was determined as usual.

	A	B
Filtered at once ... ..	9·4	4·6
Filtered after ½ hour ... ..	12	5·8

The experiment confirms the observation made in Experiment II, that *the extraction of the trypsin requires some time*. In addition it shows that more trypsin is extracted by the larger amount of casein, but this result does not seem to be quite conclusive since it was not shown in any of the cases that the extraction was finished.

### INFLUENCE OF THE AMOUNT OF WATER PRESENT

*Experiment IV.*—The charcoal-trypsin was kept at 20° for twenty-four hours, whereupon mixtures were prepared and kept at 20°.

50 charcoal-trypsin +	50 casein sol.	...	...	...	A
„ „ „ +	300 H <sub>2</sub> O	...	...	...	B
„ „ „ +	500 H <sub>2</sub> O	...	...	...	C

After three hours, when the extraction must have been finished according to Experiments I and II, the mixtures were filtered at 20°, and specimens were prepared for digestion at 37° as follows, the results of the digestion being given at the same time:—

50 filtrate A +	250 H <sub>2</sub> O +	50 cas. sol.	...	...	44·85
200 filtrate B +	100 H <sub>2</sub> O +	50 cas. sol.	...	...	44·0
300 filtrate C +	50 cas. sol.	...	...	...	44·05

The ratio between the volumes of A, B, C was 1 : 4 : 6. The digested specimens contained the filtrates in the same proportions and therefore the specimens contained the same amounts of the original charcoal-trypsin and of the casein solution. During the digestion the concentration was the same in all specimens; 50 c.c. casein solution was added in order to increase the effect. The results show, that *the amount of water present during the extraction was without influence.*

*Experiment V* was carried out with charcoal-trypsin obtained from the same reagents and in the same way as in Experiment IV. Mixtures with casein were made up as follows :—

100 charcoal-trypsin + 50 cas. sol.	...	...	...	...	A
"      "      "      + 450 H <sub>2</sub> O	...	...	...	...	B
"      "      "      + 750 H <sub>2</sub> O	...	...	...	...	C
100 charcoal-trypsin + 100 cas. sol.	...	...	...	...	D

After three hours at 20° the mixtures were filtered, and specimens made up as follows were digested at 37°, with the results given :—

25 filtrate A + 125 H <sub>2</sub> O + 50 cas. sol.	...	...	27.6
100 filtrate B + 50 H <sub>2</sub> O + 50 cas. sol.	...	...	27.45
150 filtrate C + 50 cas. sol.	...	...	27.15

The digested specimens contained amounts of the filtrates corresponding to the same amount of the charcoal-trypsin and of the casein. Moreover, the volumes and the amount of casein present were the same during the digestion. Therefore the figures obtained confirm the result obtained in Experiment IV, that *the amount of water exercises no influence.* Since it might be suggested that this result depends upon the casein having extracted the maximum of trypsin, which would not be increased by a larger amount of casein, I tried whether the larger amount of casein present in mixture D had not extracted more trypsin than the casein in the mixture A. For this purpose specimens were made up for digestion as follows, and with the results recorded :—

15 filtrate A + 5 cas. sol. + 25 cas. sol.	...	...	13.2
20 filtrate D + 25 cas. sol.	...	...	17.5

15 filtrate A correspond to the same amount of charcoal-trypsin as 20 filtrate D. In order to make the amount of casein the same in both specimens during the digestion, 5 c.c. had to be added to 15 filtrate A. To both, 25 casein sol. was added, in order to increase the effect. The figures obtained show that the larger amount of casein had extracted more trypsin, and therefore the maximum of trypsin extracted had not been reached with the lower amount of casein.

## INFLUENCE OF THE AMOUNT OF CASEIN

*Experiment VI.*—The charcoal-trypsin had been kept at  $37^{\circ}$ . Mixtures were prepared at  $37^{\circ}$  as follows:—

50 charcoal-trypsin + 250 cas. sol.	...	...	...	A
"    "    + 100 "	...	...	...	B
"    "    + 50 "	...	...	...	C
"    "    + 10 "	...	...	...	D

The mixtures all contain the same amount of charcoal-trypsin mixture, and since the ratio between the volumes is 60 : 30 : 20 : 12, volumes of the mixtures or of their filtrates, between which the same ratio is valid, must contain trypsin obtained from the same amount of charcoal-trypsin mixture. After  $1\frac{1}{2}$  hours at  $37^{\circ}$  part of the mixtures were filtered and equal volumes of the non-filtered mixtures and of the filtrates were digested at  $37^{\circ}$  with the following results:—

		Non-filtered		Filtered
60 c.c. A	...	21.55	...	20.1
30 c.c. B	...	15.9	...	15.2
20 c.c. C	...	10.65	...	10.8
12 c.c. D	...	2.55	...	2.45

The figures obtained, being the same for the non-filtered and filtered specimens (except perhaps for mixture A), show that the extraction of the trypsin was finished when the filtration took place.

In order to compare the amounts of trypsin extracted by different amounts of casein, volumes of the filtrates derived from the same amount of charcoal-trypsin mixture must be digested with the same amount of casein. For this purpose, specimens prepared as follows were digested, the results of the digestion being given at the same time:—

60 c.c. filtrate A	...	...	...	...	...	20.1
30 c.c. " B + 30 c.c. cas. sol.	...	...	...	...	...	17.8
20 c.c. " C + 40 c.c. " "	...	...	...	...	...	16.5
12 c.c. " D + 48 c.c. " "	...	...	...	...	...	8.95

The figures show that the amount of trypsin extracted increases with the amount of casein used for the extraction. Unfortunately, the amount of casein present during the digestion was not sufficient to make the results proportional to the amounts of trypsin present, and therefore no further definite conclusions can be drawn from this experiment. It is, however, obvious that the amounts of trypsin extracted were not nearly proportional to the amounts of casein used, the ratio between the amounts of casein being 25 : 10 : 5 : 1.

This being so, one must expect that for very large amounts of casein the amount of trypsin extracted should become independent of the amount of casein used for the extraction. This was the case in the following experiment:—



*Experiment VII.*—The charcoal-trypsin had been kept at 37°, and the following mixtures were prepared and kept at 37°.

10 c.c. charcoal-trypsin	+	50 c.c. cas. sol.	...	...	...	A
"	"	+ 100 "	+	10 H <sub>2</sub> O	...	B
"	"	+ 200 "	+	30 H <sub>2</sub> O	...	C

After one hour at 37° the mixtures were filtered, and volumes of the filtrates derived from the same amount of charcoal-trypsin were digested with the same amount of casein as follows:—

20 c.c. filtrate A	+	50 c.c. cas.	+	10 H <sub>2</sub> O	...	...	23'5
40 "	B	33'3 "	+	6'7 "	...	...	23'5
80 "	C	...	...	...	...	...	23'6

That the extraction was finished when the samples were filtered was ascertained by another experiment. A mixture of 30 c.c. charcoal trypsin + 300 c.c. casein was prepared and kept as above. Then 100 c.c. unfiltered mixture and 100 c.c. filtrate were digested:—

Unfiltered mixture	...	...	65'8
Filtrate	...	...	64'4'

### DISCUSSION OF THE RESULTS

The above experiments give, no doubt, a very strong support to the view that the proteins combine with trypsin before they are broken up by the same. As the experiments were arranged, the casein was made to extract trypsin which had been previously adsorbed by charcoal. During the process of extraction, therefore, the trypsin was acted upon by two substances endowed with the power of taking it up—charcoal and casein. The final distribution of casein between the charcoal and the trypsin was reached in, as a rule, less than half an hour after the casein had been added to the charcoal-trypsin mixture. Both charcoal and casein take up trypsin in some proportion to the amount of substances present, and therefore the final distribution of the trypsin will be the effect of some kind of mass action

1. With regard to the above experiments, it may be mentioned that it has been found to be very difficult completely to remove charcoal from a fluid that contains casein. Even after repeated filtration traces of charcoal occur in the filtrate. Evidently these traces can be of no influence, in case the extraction was finished when the filtration took place and if at the same time the following digestion is carried out at the same temperature as the extraction and filtration (Experiment VI, VII). In other cases the figures obtained from the filtered specimens might be a trace too high, which would, however, in no instance affect the conclusions drawn. The amount of digestion taking place before and during the filtration, being the same or approximately the same for filtered and non-filtered specimens, does not influence the results.

On the other hand, the effect of this mass action can not be the same as that of Guldberg-Waage's law for real solutions, because in the case of real solutions the combinations formed are reversible and, therefore, the final equilibrium will be the same independently of the way in which it has been arrived at.

In the case of charcoal and trypsin, on the contrary, the trypsin adsorbed by degrees becomes fixed to the charcoal and only a small portion of it can be extracted by added casein, less being extracted the longer the time and the higher the temperature for the interaction between the charcoal and trypsin before the casein is added.<sup>1</sup> The final distribution of the trypsin, therefore, depends very much upon the way in which it has been arrived at.

#### SUMMARY

1. Casein is capable of extracting trypsin adsorbed by charcoal. The extraction requires some time to be finished (Experiment III). Sooner or later (as a rule in less than half an hour at 20°) the extraction comes to an end as borne out by the fact that a filtered specimen gives the same tryptic effect as the non-filtered material (Experiments VI, VII). The end of the extraction can also be recognised by the fact that specimens filtered after different intervals of time contain the same amount of trypsin (Experiments I, II).

2. The final amount of trypsin extracted is larger the higher the temperature (Experiments I, II).

3. For small amounts of casein the final amount of trypsin extracted increases with the amount of casein (Experiment VI). For large amounts of casein the amount of trypsin extracted is independent of variations in the amount of casein (Experiment VII).

4. The amount of water present is without influence upon the final amount of trypsin extracted (Experiments IV, V). This rule holds good even for small amounts of casein (Experiment V).

The results support the view that proteins combine with trypsin before they are broken up by the same.

1. *Bio-Chemical Journal*, I, 489, 1906.

## THE DIASTATIC FERMENT IN THE TISSUES IN DIABETES MELLITUS

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A striking fact observed in experimental diabetes after removal of the pancreas is the great diminution in the hepatic glycogen, notwithstanding the hyperglycaemia. Observations on the amount of glycogen present in the livers of diabetic patients are difficult to obtain and few in number; but the quantity present appears to be less than normal. On Bernard's hypothesis, the conversion of glycogen into dextrose is carried out by a diastatic ferment normally present in the liver. It is possible that the conversion of dextrose into glycogen also is effected by an enzyme, which might be either the diastatic ferment acting in a reverse way, or a different ferment altogether. The apparent inability of the liver in diabetes to store glycogen might be due to the absence of the necessary ferment. We have investigated the livers, blood and muscles of diabetic patients and of depancreatised cats, in order to ascertain whether they possess the normal diastatic ferments; and this we have found to be the case. The future question, whether it can be shown that the normal liver possesses a ferment which can act synthetically upon dextrose, and if so, whether this ferment is present or absent in diabetes, is under investigation.

### METHODS

*Preparation of the tissue extracts.* Pavy has shown that pieces of liver, kept under alcohol for months, still contain an enzyme capable of converting glycogen into sugar. Our method of obtaining the ferment is based upon this observation, and is a modification of that employed by Pick. Miss Tebb has shown that the enzyme acts not only upon glycogen but also upon starch; and in most of our

experiments we have used Kahlbaum's pure soluble starch, as being more convenient to work with. In a few experiments we have used solutions of Merck's pure glycogen and obtained the same results as when using soluble starch.

Portions of liver, obtained as soon as possible after death, were minced and placed under five times their volume of 95 per cent. alcohol for several weeks. In the animal experiments, the liver was washed out and freed from blood before being minced and placed under alcohol. The alcohol finally was filtered off, and the liver washed with alcohol until the washings were free from sugar. The liver then was dried at  $37^{\circ}$  C., powdered and rubbed up with sand and water; some toluol was added, and the extraction was continued at  $37^{\circ}$  C. for twenty-four hours, during which time the flask was frequently shaken. The extract was filtered and yielded a clear yellow filtrate, which gave no colour reaction with iodine, and which contained small quantities of protein and sugar. Known quantities of this filtrate were incubated with a solution of soluble starch for twenty-four hours; an abundance of toluol was added. The solutions were then boiled in order to coagulate the protein and drive off the toluol; they were cooled, made up to a known volume and filtered. The filtrate was tested with iodine, and the amount of copper reducing substance present in it was estimated by Pavy's method, and the result is expressed as dextrose.

The blood used for extraction was received, during life, direct into alcohol. The subsequent procedure was identical with that described above in the case of the liver.

The muscle extract was made in the following way:—

In the animal experiments the muscles were washed, directly after death, as free from blood as possible by means of normal salt solution injected through the aorta. In the case of diabetic patients the muscles were not freed from blood. Portions of muscle were minced and pounded, first with sand and a little water, and then with Kieselguhr, until a nearly dry powder was obtained. This was exposed to pressure in a Buchner's press, and the muscle juice collected. The juice was then examined for the presence of ferment.



The extract of pancreas added to the other extracts in certain experiments corresponded to that described by Cohnheim, and was essentially a boiled watery extract of pig's pancreas, which had previously been minced and pounded up with sand. The strength of the extract was such that 2 c.c. of the solution contained the extract of about 1 gr. pancreas.

### EXPERIMENTS

The presence in the liver, under normal conditions, of an enzyme capable of converting starch or glycogen into dextrose is so well established, that we merely examined a few livers taken from normal animals and from the post mortem room in order to demonstrate, firstly the effectiveness of the method of extraction, and secondly the existence of the enzyme in the livers twenty-four hours or more after death.

Two of the post mortem cases are recorded here :—

*Case I.*—Liver, obtained twenty-four hours after death, from a patient who died two hours after an accident which fractured his vertebral column. 25 c.c. of the liver extract were added to 30 c.c. of 1 per cent. solution of soluble starch, toluol was added, and the whole was incubated at 37° C. for twenty-four hours. The control flask was boiled for several minutes before the toluol was added. At the end of twenty-four hours the flasks were boiled, cooled, made up to 100 c.c., and filtered. The following table gives the result obtained :—

		Iodine Test	Sugar by Pavy
Control ...	...	Blue	0.06 gr.
Unboiled flask ...	...	Violet	0.18 gr.

*Case II.*—Patient died of a cerebral tumour; liver obtained forty-eight hours after death. Experiment carried out in the same way as above.

		Iodine Test	Sugar by Pavy
Control ...	...	Blue	0.075 gr.
Unboiled flask (1)	...	Violet	0.18 gr.
„ „ (2)	...	Violet	0.20 gr.

As regards the blood of normal men and animals, it has been shown by Ascoli and Bonfanti that a diastatic ferment is often present; and we have confirmed this in the case of the dog by the above method.

The muscle juice of normal animals possesses a feeble diastatic power. An experiment is recorded later which shows this effect in the dog.

(a) *The liver extract in diabetes.* We have examined the livers of four diabetic patients and of four depancreatized cats. The full clinical histories of the patients have been given in a previous paper (p. 439); the cases are not numbered consecutively, but have received the same numbers in this paper as they had in the previous one. The cats had undergone almost complete removal of the pancreas, and showed in each case well marked symptoms of diabetes; their urine contained an abundance of sugar and gave a feeble reaction with ferric chloride.

The method employed was the same in all cases. Thirty c.c. of liver extract were added to 40 c.c. of 1 per cent. solution of soluble starch; toluol was added, and the mixture incubated for twenty-four hours at 37° C. In some experiments a second flask contained the same mixture, with the addition of 10 c.c. of pancreatic extract, which corresponded to about 5 grs. of pancreas. The control flask was boiled before the addition of toluol. In some cases a fourth flask, containing liver extract with water and toluol, was incubated. The following table gives the results obtained:—

Case	Liver extract and water			Boiled control	Liver extract and starch at 37°	Liver extract, starch, and pancreatic extract at 37°
II. Diabetic Coma						
Iodine ...	...	...	No colour	Blue	No colour	—
Sugar by Pavy	...	...	0.05 gr.	0.05 gr.	0.24 gr.	—
III. Diabetic Coma						
Iodine ...	...	...	No colour	Blue	Violet	—
Sugar by Pavy	...	...	0.06 gr.	0.06 gr.	0.26 gr.	—
V. Diabetes and Phthisis						
Iodine ...	...	...	—	Blue	Violet	Violet
Sugar by Pavy	...	...	—	0.09 gr.	0.21 gr.	0.20 gr.

Case			Liver extract and water	Boiled control	Liver extract and starch at 37°	Liver extract, starch, and pancreatic extract at 37°
VI. Diabetes and Septicaemia						
Iodine ...	...	...	No colour	Blue	Violet	—
Sugar by Pavy ...	...	...	Trace	Trace	0·18 gr.	—
Cat A						
Iodine ...	...	...	—	Blue	Violet	Violet
Sugar by Pavy ...	...	...	—	Trace	0·10 gr.	0·12 gr.
Cat B						
Iodine ...	...	...	—	Blue	Violet	Violet
Sugar by Pavy ...	...	...	—	o	0·18 gr.	0·18 gr.
Cat E						
Iodine ...	...	...	No colour	Blue	Violet	—
Sugar by Pavy ...	...	...	o	o	0·12 gr.	—
Cat F						
Iodine ...	...	...	No colour	Blue	Violet	Violet
Sugar by Pavy ...	...	...	o	o	0·11 gr.	0·10 gr.

The presence of sugar in some of the boiled controls indicates that the corresponding livers contained both glycogen and a ferment capable of converting glycogen into sugar.

(b) *The muscle juice in diabetes.* Muscle was obtained from two patients, Cases II and III in the previous table, and from cat E. The same method was used throughout. Eight c.c. of muscle juice were added to 20 c.c. of 1 per cent. solution of soluble starch; toluol was added, and the mixture incubated for thirty hours at 37° C. A second flask contained the same solutions together with 10 c.c. of pancreatic extract. The control flask was boiled for several minutes before the toluol was added. At the end of thirty hours the flasks were boiled, cooled, made up to 100 c.c., and filtered. The following table of results contains, also, those obtained with the muscle of a normal dog.

Case		Boiled control	Muscle juice and starch at 37°	Muscle juice, starch and pancreatic extract at 37°
II. Diabetic Coma				
	Iodine ...	... Blue	Violet	Violet
	Sugar by Pavy	... o	0·11 gr.	0·12 gr.
III. Diabetic Coma				
	Iodine ...	... Blue	Violet	Violet
	Sugar by Pavy	... o	0·05 gr.	0·05 gr.
Cat E				
	Iodine ...	... Blue	Blue	Violet
	Sugar by Pavy	... o	0·03 gr.	0·05 gr.
Normal Dog				
	Iodine ...	... Blue	Violet	—
	Sugar by Pavy	... o	0·04 gr.	—

(c) *The blood extract in diabetes.* Blood was obtained during life from three diabetic patients, and from one depancreatized cat. One of the patients was Case II in the previous tables, and his blood was obtained when he was in coma. The two other patients were suffering from severe diabetes. When their blood was taken they were being treated by secretin given by the mouth. Their full clinical histories have been given in a previous paper (p. 433), where they are referred to as Cases II and III. It may be mentioned that Case III died of diabetes in November, 1906, eight months after our observations upon him were made.

The blood was treated and its diastatic activity observed by the method already described, so that it is unnecessary to give more than a table showing the results obtained.

Case	Boiled control	Blood extract and Starch at 37°
(1) Diabetic coma.—Case II		
Iodine	Blue	No colour
Sugar by Pavy	Trace	0·11 gr.
(2) Severe diabetes.—Case II		
Iodine	Blue	Blue and Violet
Sugar by Pavy	Trace	Too little to estimate
(3) Severe diabetes.—Case III		
Iodine	Blue	Blue and Violet
Sugar by Pavy	Trace	Too little to estimate
(4) Cat E		
Iodine	Blue	Violet
Sugar by Pavy	o	0·15 gr.



The almost complete absence of a diastatic ferment seen in the two cases of severe diabetes, has also been observed by Ascoli and Bonfanti in some healthy human beings and animals.

#### CONCLUSIONS

The livers and muscles of diabetic patients and of depancreatized cats contain a diastatic ferment. No attempt was made to ascertain whether the amount of enzyme present was greater or less than that found in normal animals. In two cases a diastatic ferment was found in the blood, and in two others it was absent. No evidence was obtained that the activity of the diastatic ferment of liver or muscle is increased by the addition of a boiled extract of pancreas.

Part of the expense of this investigation was defrayed by a grant from the Royal Society.

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## ON THE RÔLE OF PHENOLS, TANNIC ACIDS, AND OXYBENZOIC ACIDS IN CORK FORMATION

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Tannins, phenols and oxybenzoic acids are very generally present in plant tissues. Among the more commonly occurring phenols are phloroglucinol, resorcinol, and hydroquinone; of oxybenzoic acids, gallic, salicylic and protocatechuic are commonly found. Some of these substances are widely distributed particularly tannins and phloroglucinol (1). If present in considerable quantity they generally act as poisons, but in some plants, for instance, *Loxopterygium Lorenzii*, Griesb., *Eucalyptus occidentalis*, Endl., *Caesalpinia coriaria*, Willd., and *C. brevifolia*, Baill., tannins are present in very large proportions. Pfeffer (2) has suggested that these substances being contained within the vacuole of the cell, fail to exercise a poisonous influence, not being capable of penetrating the protoplasmic boundary of the vacuole. When plants are fed with glucosides an injurious influence is often exerted owing to the liberation of the oxyaromatic radicles. It has been supposed that tannins may serve as reserve food materials, but this is still very doubtful. This question will be re-opened in another paper dealing with the function of tannic acids in the plant economy.

It is generally believed that tannins and other aromatic compounds play an important part in the lignification of the wood elements. Very little is known, however, with certainty, as to the function of these bodies in plants.

When tannins, phenols and oxybenzoic acids are treated with formaldehyde and a little hydrochloric acid, condensation products are precipitated. These have been studied by Bayer (3), Kleeberg (4),

Caro (5), Möhlau and Kahl (6), Nierenstein (7), Stiasny (8), and others. They are mixtures of diphenylmethaneoxybenzoic and aurinoxycarbonic acids. Stiasny in particular has studied the condensation products of formaldehyde with various tannic acids. All these products are powders, varying in colour from white through pale yellow to reddish brown and deep brown.

In experimenting with these condensation products we found that they could be produced from gallic acid, pyrogallol, protocatechuic acid, resorcinol, phloroglucinol, salicin, tannic acid, and quebrachotannic acid. All the previous workers seem to have used hydrochloric acid, but we have found that similar substances are precipitated by using phosphoric, acetic, and formic acids, and more slowly by passing carbon dioxide through the mixture of formaldehyde and the phenol, tannic or oxybenzoic acid. A study of the reactions of these bodies has shown a remarkable similarity to those given by cork. Thus, they are insoluble in copper ammonium hydroxide (Schweizer's reagent) and in concentrated sulphuric acid, but dissolve readily in strong potassium hydroxide, giving a yellow or deep red-brown solution.

This suggested that in the formation of cork similar condensation products may play an important part. It is known that the required phenols, tannins and oxybenzoic acids are present in the plant and there is but little doubt that formaldehyde is also present; indeed, in some cases it is quite possible to demonstrate it.

An examination of a number of living plants has been made in order to trace the distribution of the tannic acid, phenols and oxybenzoic acids in the stem. The usually adopted method of testing for tannins in plants is by the use of ferric chloride, which gives a blue-black or green-black colour; with ferric chloride gallic acid gives a similar colouration. Potassium bichromate is also used and is said to give a dark brown colouration with tannic acids. These methods were employed by us. In addition, it was found that a most useful reagent for microchemical work is potassium cyanide. This gives a clear pink colour in the presence of gallic acid. Young (9) has used this reagent with success in chemical analysis, but we believe that it

has not generally been used in microchemical testing. It is also of great interest to note that contrary to the generally accepted belief that a brown colour is given by the action of potassium bichromate on tannic acid in microchemical work, we find that while gallic acid gives this reaction immediately, tannic acid fails to do so, or only develops a brown colour slowly.

Other reagents that have been used are gold chloride and ammonia, but for microchemical testing they have been less useful than potassium cyanide and ferric chloride.

The following plants have been examined :—

*Sambucus nigra*, L.

Potassium cyanide. A little red colouration in the cells in the immediate neighbourhood of the cork. (Fig. 1).

Ferric chloride. A little blue-black in the same positions.

Potassium bichromate. A little browning in the cork.

*Ribes Grossularia*, L. This plant has deeply seated cork.

Potassium cyanide. A red colour in the cells both outside and inside the cork. The walls of the cells bounding the cork layer internally also gave a strong red colour. (Fig. 2).

Ferric chloride. A tinge of blue-black in the same positions.

Potassium bichromate. A rich brown colour.

*Acer Pseudoplatanus*, L. This plant has deeply seated cork.

Potassium cyanide. Well developed red colour on both sides of the cork and in the external cortex. (Fig. 3).

Ferric chloride. Blue-black colour in the same positions. (Fig. 4).

Potassium bichromate. A deep brown colour.

*Fuchsia*. In this plant the cork cells are of two kinds, large and small, alternating with one another in a radial direction.

Potassium cyanide. Red colour in the cells internal to the cork and in the small cells of the cork itself ; also a little in the cells external to the cork. (Fig. 5).

Ferric chloride. Well developed blue-black colour in the same positions. (Fig. 6).

Potassium bichromate. Extensive browning.



*Ilex Aquifolium*, L.

No cork present.

Potassium cyanide. No red colour.

Ferric chloride. No blue-black colour.

Potassium bichromate. No brown colour.

*Ulmus campestris*, L.

Potassium cyanide. Much red colour outside the cork.

Ferric chloride. Much blue-black colour outside the cork.

Potassium bichromate. No colouration.

*Helianthus* sp.

A young stem with no cork formation was used.

Potassium cyanide. No colour.

Ferric chloride. No colour.

Potassium bichromate. No colour.

*Cheiranthus Cheiri*, L.

Potassium cyanide. Some red colour in the neighbourhood of the cork.

Ferric chloride. A little blue-black colour particularly outside the cork.

Potassium bichromate. A brown colour.

*Ribes rubrum*, L.

Potassium cyanide. Deep red colouration in the cortex and inside the cork.

Ferric chloride. A little blue-black in neighbourhood of the cork.

Potassium bichromate. Brown colour in the same positions.

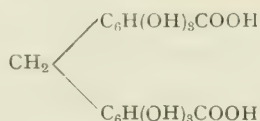
In the examination of the plants described, in every case where cork was found, a colouration of the cells in the immediate vicinity was shown in the presence of potassium cyanide or ferric chloride, and usually also with potassium bichromate, indicating the presence of gallic or tannic acids. In the two plants which showed no reaction with ferric chloride, or potassium cyanide, namely, the Sunflower and the Holly, no cork was present.

## CHEMICAL EXAMINATION OF THE CORK

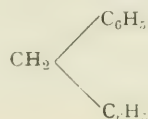
Cork shavings, probably the produce of *Quercus Suber*, L., were extracted with water. The aqueous solution was tested with ferric chloride and with potassium cyanide and gave a blue-black with the former, and a red with the latter reagent. The aqueous solution was acidified with sulphuric acid and then extracted with ether. After distilling off the ether, small crystals of gallic acid were obtained which melted at  $236-237^{\circ}$  C., the melting point of gallic acid being given as  $240^{\circ}$  C. The presence of gallic acid in the cork was thus demonstrated. Chevreul (10) in 1807 and 1815, stated that he had obtained gallic acid by extracting cork with water, and his statement is thus confirmed.

The cork was next extracted with alcohol and the extract was not examined. The remaining cork was then extracted for two hours with a 30 per cent. solution of potassium hydroxide on a steam bath. It was then filtered through glass-wool. The extract was neutralised with sulphuric acid, when a brownish yellow precipitate was formed. This was distilled over zinc dust and diphenylmethane was obtained, small crystals of which from chloroform gave a melting point  $25.5^{\circ}$  C. The powder was also distilled in coal gas with the result that pyrogallol was formed. By fusion with alkalis, protocathechuic acid was produced and another substance, of which no crystals could be obtained.

The condensation products of gallic acid with formaldehyde have been thoroughly studied by Möhlau and Kahl (6), who have shown them to consist of a mixture of four different substances one of which is hexaoxydiphenylmethanedicarbonic acid. This has the following constitution :—

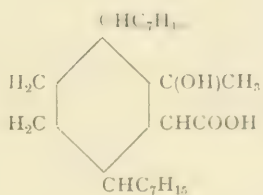


On reduction this should give diphenylmethane :—



To prove this the condensation product of gallic acid with formaldehyde was distilled with zinc dust and diphenylmethane resulted, the crystals from chloroform having a melting point of  $25^{\circ}\text{C}$ .

The fact that the cork examined by us yields diphenylmethane strongly supports our belief that condensation products of a similar nature to those produced artificially by the action of formaldehyde on the phenols, tannic acid and oxybenzoic acids play a part in cork formation. For these condensation products the name *phellemic acids* is suggested. The name phellonic acids which was first chosen, has been used previously by K  gler (11), Thoms (12), Hientz (13), Gilson (14), and v. Schmidt (15), for a substance extracted from cork by chloroform. For this phellonic acid v. Schmidt has suggested the constitution :—



Evidently this could not yield diphenylmethane and hence it is not related to our phellemic acids.

The facts related above have led us to attribute to tannic acids, phenols, gallic acid and other oxybenzoic acids an important r  le in the formation of cork. The presence of substances of this class has been clearly shown in close association with the cork wherever this tissue was found. It has been shown that substances giving cork reactions are precipitated from tannic acids, phenols and oxybenzoic acids by means of formaldehyde in presence of various acids, and products having the same mother substance as these condensation products have been obtained from cork itself.

Our theory then is that the tannic and gallic acids, and substances of this nature are acted upon in the plant by formaldehyde and acids, and are precipitated in the walls of the cork cells.

## SUMMARY

1. Condensation products which give reactions similar to those given by cork (insolubility in copper ammonium hydroxide and in concentrated sulphuric acid; solubility in potassium hydroxide) are precipitated by the action of hydrochloric, phosphoric, acetic, and formic acids, and of carbon dioxide on a mixture of formaldehyde and a phenol, tannic acid, or oxybenzoic acid.

2. The condensation product of gallic acid yields diphenylmethane when reduced with zinc dust.

3. Tannic and oxybenzoic acids are present in the plant in close association with the cork.

4. From cork itself was extracted gallic acid and a substance resembling the condensation products prepared artificially. This last substance when reduced with zinc dust yielded diphenylmethane.

5. Probably the condensation products of formaldehyde with tannic and oxybenzoic acids are formed in the plant and precipitated at an early stage in the cell walls of the cork.

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## EXPLANATION OF PLATE

- Fig. 1. Transverse section of stem of *Sambucus nigra*, L., treated with potassium cyanide.  
 Fig. 2. Transverse section of stem of *Ribes Grossularia*, L., treated with potassium cyanide.  
 Fig. 3. Transverse section of stem of *Acer Pseudoplatanus*, L., treated with potassium cyanide.  
 Fig. 4. Transverse section of stem of *Acer Pseudoplatanus*, L., treated with ferric chloride.  
 Fig. 5. Transverse section of stem of *Fuchsia* treated with potassium cyanide.  
 Fig. 6. Transverse section of stem of *Fuchsia* treated with ferric chloride.



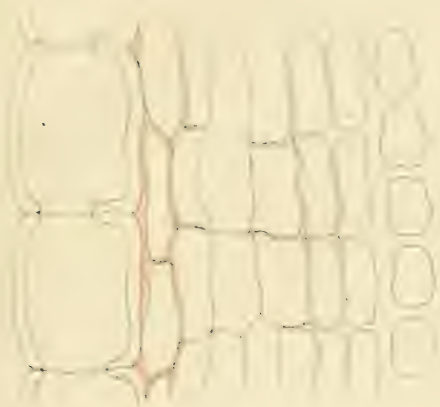


Fig 1

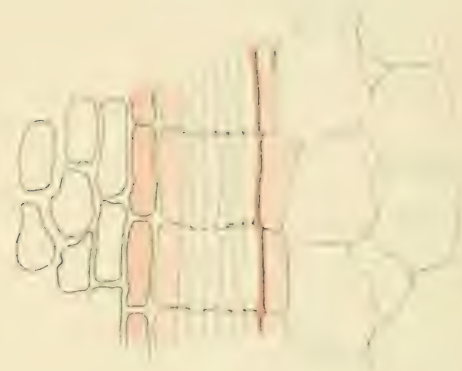


Fig 2

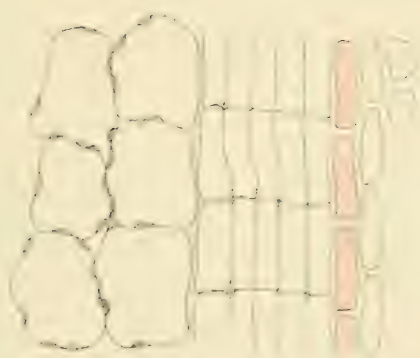


Fig 3

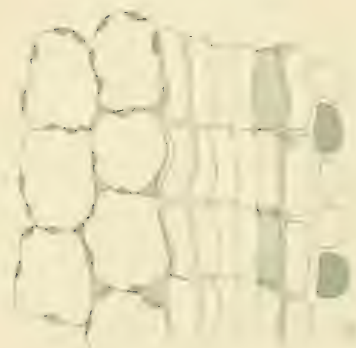


Fig 4



Fig 5

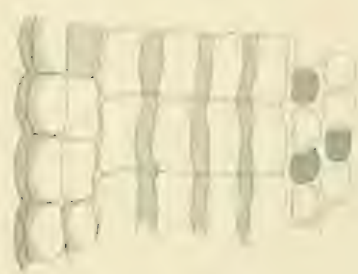


Fig 6



# IMPROVED METHOD FOR THE DETERMINATION OF SUGAR IN BLOOD AND OTHER TISSUES, WITH A CONSIDERATION OF THE CONDITION OF THE SUGAR IN BLOOD

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Up to the present, many methods have been devised for the determination of sugar in blood and other tissues. These methods, however, are all the same in principle, depending on the removal of the protein from the solution and the subsequent determination of the sugar by reduction or fermentation. Methods for the determination of sugar in blood depending on these principles have been devised and employed, among others, by Abeles, Schenck, Weyert, Reid, Schmidt-Mülheim-Hofmeister, Pavy, and a comparison of these methods has been made by Seegen.<sup>1</sup>

Where only a determination of the total sugar in the blood is desired these precipitation methods are probably satisfactory, provided that the method is carefully carried out and a sufficient number of determinations is made. These methods, however, are insufficient where a knowledge not only of the total sugar is desired, but some indication also of the condition in which this sugar exists in the blood or tissue. It is obvious, for example, that should a part of the sugar in blood exist in loose chemical combination (adsorption) with the protein or other material of the blood, such methods will fail completely to discriminate between such sugar and that existing in the free state.

The most general method depending on precipitation is that described by Schenck,<sup>2</sup> and modified by others. This method consists in precipitating the protein of the blood with an acid solution of

1. Abeles, *Zeitschr. f. Physiol. Chem.*, XV, 495. Schenck, *Pflüger's Arch.*, LV, 203-211. Weyert, *Dubois Reymond's Arch.*, 1891, 187. Reid, E. W., *Journ. Physiol.*, XX, 316-321. Schmidt-Mülheim-Hofmeister, *Dubois Reymond's Archiv.*, 1879, and *Zeitschr. f. Physiol. Chem.*, IV, Pavy, *Proc. Roy. Soc. XXXII*, 418 (1881), also *Brit. Med. Journ.*, I (1896). Seegen, *Centralblt. f. Physiol.*, 1892, p. 501 and 624.

2. *Pflüger's Archiv.*, LV, 203-211.

mercuric chloride, filtering and precipitating the excess of mercury in an aliquot portion of the clear liquid with hydrogen sulphide. The filtrate obtained after separation of the mercuric sulphide is boiled till free from hydrogen sulphide, neutralised, and the sugar determined by reduction. The use of such a chemical agent as mercuric chloride in acid solution is undesirable on the grounds mentioned above. Further, the assumption that the sugar is equally distributed between precipitate and mother-liquor, in other words that there is no mechanical retention of the sugar, is not warranted, as Seegen points out.

Where the after removal of hydrogen sulphide is effected by boiling, the hydrolysis of any hydrolysible sugar is certain to follow, and it is further probable that the long continued boiling necessary to remove the last traces of hydrogen sulphide will result in a partial destruction of the sugar. Although we have not determined the limits of experimental error by this method, we have, nevertheless, found that it has several of the above-mentioned disadvantages.

Other precipitation methods, for example the zinc acetate or the method using alcohol, are unsatisfactory because by their use the normal condition of the blood is destroyed and there is no proof that the use of such reagents does not produce secondary changes in the blood.

In any satisfactory method of determining the sugar in blood under varying conditions, the blood must be collected and treated with aseptic precautions as far as possible, and the sugar determined in its natural condition in the blood, chemical reagents, boiling, etc., being avoided where secondary changes are liable to occur.<sup>1</sup> A brief consideration of the many methods up to the present employed for the determination of sugar in blood will show, that in none of these is the second condition above mentioned fulfilled.

For the purpose of investigating the nature of the sugar in blood under various conditions, the method of dialysis has been found by us to yield satisfactory results. This method, as far as we are aware, has never been hitherto successfully employed in determining the

1. It is at present hard to say how far the results obtained by Lepin  and others, in support of a theory of glycolytic fermentation "*in vitro*" may not be explained on one of the above grounds.



nature and amount of the sugar in blood, although Hédon<sup>1</sup> has employed it for isolating and identifying the sugar, while Schenck<sup>2</sup> and Arthus<sup>3</sup> have employed it, unsuccessfully however, in attempts to prove whether or not the sugar in the blood exists in the free state or in combination with the protein.

After a number of preliminary experiments, the following method was adopted by us. The blood, which had been previously collected in a sterile vessel, was defibrinated aseptically, then 50 c.c. of it was drawn off and introduced into a dialysing tube of ordinary sausage-skin form, contained in a wide-mouthed bottle of about 500 c.c. capacity, provided with a tightly fitting glass stopper. Every piece of apparatus used was previously sterilized, and all the subsequent operations were carried out with aseptic precautions. In the early experiments the blood or serum was dialysed into water, but later, saline was found to be better.

In all the experiments recorded a measured quantity (300 to 450 c.c.) of 0.9 per cent. sodium chloride was used. The process of dialysis was continued about forty to forty-five hours, experiments having shown that before this time equilibrium had been reached.<sup>4</sup> During this interval the whole apparatus was kept in an ice-chamber.

The volume of the saline outside the dialysis-tube at the end of the experiment was accurately measured, the bottle and dialysis tube being then rinsed with water. The saline and washings were then brought to boiling, and when this point had been reached boiling Fehling's solution considerably in excess of that required for complete oxidation of the sugar was added, and the whole kept in gentle ebullition for the space of five minutes. This procedure was found to be necessary, otherwise the precipitate of cuprous oxide was thrown down in such a fine state of division that it remained partly suspended in the mother liquor, and was with difficulty prevented from passing through the filter. By the above method the precipitate came down in a granular form and no difficulty was experienced in filtering.

1. Hédon, *Compt. Rend. Soc. Biolog.*, 50, 510.

2. Schenck, *Pflüger's Archiv.*, XLVII, 621.

3. Arthus, *Zeitsch. f. Biolog.*, 34, 438.

4. On one occasion, where dialysis was inadvertently discontinued after half-an-hour, it was found that approximately 10 per cent. of the sugar had already dialysed out.

The cuprous oxide was collected on a tared Gooch crucible, washed thoroughly with distilled water, dried and ignited, the reduction being determined by the weight of the cupric oxide so obtained.

For the purpose of determining the accuracy of the method numerous experiments have been carried out, some with the serum alone and some with the defibrinated blood. In other experiments the entire dialysis apparatus was brought to 100° immediately after the introduction of the blood, and maintained at that temperature until coagulation was effected. This last process invariably gave higher figures for the sugar, but that this increase is not due to the action of bacteria in the uncoagulated blood, but is more probably the result of a decomposition of some loosely-combined sugar in the blood, we have great reason to believe, as will be shown in a later paper in which we propose to describe the action of carbon dioxide and other anaesthetics on the blood.

The following experiments serve to show the accuracy of the method.

*Experiment I.*—Pig's blood drawn under aseptic precautions was immediately defibrinated and centrifugalised for 1½ hours. The serum was then separated from the corpuscles and each was thoroughly mixed in sterile beakers. The dialysis of the whole blood, serum and corpuscles, was then carried out as already described, 450 c.c. of saline being used, and the whole dialysed for forty-three hours.

Results :—

No.	Nature	Final volume of Saline	CuO	Percent. Dextrose	Mean value of Dextrose
1	Serum with 3 drops xylol added ... ..	442 c.c.	0.0998 gm.	0.1027 %	0.1040 %
2	„ „	444 c.c.	0.1022 „	0.1046 %	
3	„ „	444 c.c.	0.1023 „	0.1047 %	
4	Corpuscles, with 3 drops xylol added ... ..	442 c.c.	0.0028 „		0.0474 %
5	„ „	442 c.c.	0.0054 „		
6	„ „	443 c.c.	0.0025 „		
7	Whole blood, 3 drops xylol added ... ..	441 c.c.	0.0466 „	0.0480 %	
8	„ „	442 c.c.	0.0463 „	0.0476 %	
9	„ „	444 c.c.	0.0456 „	0.0467 %	

This experiment indicates the data necessary for calculating the results. For the conversion of copper oxide into dextrose the factor employed is 0.4545. For the sake of shortness only the percentage of dextrose will be given in subsequent experiments.

*Experiment II.*—The whole blood in this experiment was coagulated in the dialysing tube at 100°, soon after being drawn. The serum, on the other hand, eight hours later. Blood dialysed for forty hours into 450 c.c. saline.

Percentages of dextrose in :—

SERUM			WHOLE BLOOD		
1	2	3	1	2	3
0.1247%	0.1248%	0.1248%	0.0676%	0.0644%	0.0722%
Mean = 0.1248%			Mean = 0.0681%		
CORPUSCLES					
1	2	3			
0.0043%	0.0046%	0.0045%			
Mean = 0.0045%					

*Experiment III.*—Dialysis continued for forty-two hours, and blood or serum uncoagulated throughout.

Percentages of dextrose :—

SERUM		WHOLE BLOOD		CORPUSCLES	
1	2	1	2	1	2
0.1187%	0.1187%	0.0607%	0.0603%	0.0002%	0.0009%
Mean = 0.1187%		Mean = 0.0605%			

*Experiment IV.*—Pig's blood dialysed for forty hours. Blood uncoagulated. Percentage of dextrose :—

SERUM		WHOLE BLOOD		CORPUSCLES	
1	2	1	2	1	2
0.1189%	0.1202%	0.0665%	0.0602%	0.0004%	0.0006%
Mean = 0.1195%		Mean = 0.0633%		Mean = 0.0005%	

Although other experiments have been made, nevertheless these suffice to show the degree of accuracy which can be obtained by this method, and further throw some light on the distribution of sugar in the blood. They show the probability of entire absence of sugar from the corpuscles, the reduction observed with them being easily accounted for in every case by the sugar in the admixed serum, and Experiment Number II in particular illustrates this important point. Here, the whole blood in which all glycolytic and bacterial action had

been completely stopped soon after collection, was found to contain no more sugar than would be accounted for by the serum if we accept the relative proportion of serum to corpuscles as about 60:40. If the absence of sugar in the corpuscles is to be explained by a rapid destruction of the same, then the proportion of sugar in the whole blood to that in the serum, as found in this experiment, ought to have been much higher.<sup>1</sup>

For the purpose of further investigation we have recentrifuged corpuscles with a volume of saline equal to that of original serum, and in this way have failed to find even a trace of sugar in the resulting corpuscles. Furthermore, in another experiment, we have determined the amount of sugar in whole blood coagulated three hours after collection as compared with the same blood coagulated immediately on being drawn. The loss of sugar in this time did not amount to more than one per cent., a difference well within the limits of experimental failure and hardly warranting a theory of glycolytic action.

In the following experiments an attempt has been made to demonstrate the increase in the amount of sugar obtainable by this method after coagulation.

*Experiment IV.*—With pig's blood, dialysed, against 450 c.c. 0.9 saline for forty-six hours.

Sugar percentage in whole blood :—

CONTROL UNCOAGULATED			BLOOD COAGULATED		
1	2	3	1	2	3
0.0530%	0.0523%	0.0521%	0.0561%	0.0554%	0.0559%
Mean = 0.0525%			Mean = 0.0558%		

*Experiment V.*—With pig's serum dialysed for forty hours.

Percentage of sugar :—

CONTROL SERUM		COAGULATED SERUM	
1	2	1	2
0.1189%	0.1202%	0.1277%	0.1195%
Mean = 0.1195%		Mean = 0.1236%	

1. Lepin  and Boulud (*Compt. Rend.*, 141 p. 175) claim to have found as much as two-fifths of the sugar in the whole blood in the corpuscles. They centrifuged the blood, however, for only ten minutes and it is therefore doubtful how far complete separation of the serum and corpuscles had been effected.



*Experiment VI.*—Pig's serum dialysed forty-three hours.

Percentage of sugar :—

CONTROL SERUM			COAGULATED SERUM		
1	2	3	1	2	3
0·0763%	0·0761%	0·0735%	0·0843%	0·0823%	0·0821%
Mean = 0·0753%			Mean = 0·0829%		

*Experiment VII.*—Sheep's serum dialysed for forty-two hours against 300 c.c. saline.

Percentage of sugar :—

CONTROL SERUM		COAGULATED SERUM	
1	2	1	2
0·0609%	0·0620%	0·0700%	0·0682%
Mean = 0·0614%		Mean = 0·0691%	

It seems to us impossible to explain the constancy of these and many other experiments made by us on any other ground than that of an existing affinity between the sugar and some other constituent of the serum, an affinity which is known to exist in the case of lecithin and glucose and which may equally well exist between proteins and glucose. That these differences are not the result of bacteriolytic or glycolytic action, as some might argue, we have further evidence to show. We have found, for example, that not only can this increase be obtained by complete coagulation of the blood or serum, but that a similar rise in the sugar obtainable by dialysis is always to be observed when the blood or serum has been previously treated with carbon dioxide, and further, that this rise can again be annulled if the carbon dioxide is displaced in the serum by oxygen. These facts cannot be explained on any theory of glycolysis or bacterial action. In further support of the existence of some form of loose chemical combination between glucose and some of the constituents of the serum, preliminary experiments with artificially prepared lecithin-sugar dissolved in saline, dialysed under similar conditions, have led to analogous results.<sup>1</sup>

1. Details of the action of carbon dioxide and anaesthetics in general on the sugar-content of blood and other tissues, will shortly be published. We intend, also, to follow up the remarkable behaviour of lecithin sugar in regard to anaesthetics, and to extend these investigations to the behaviour of glucose in presence of protein.

In conclusion, we carried out several experiments in order to compare the values obtained by our method with those by precipitation methods. We have selected the method of Schenck, described in the earlier part of this paper, and have followed his methods as nearly as possible. The coagulation of the proteins was effected by treating the blood or serum with a hydrochloric acid solution of mercuric chloride. This process, as one would naturally suppose, is bound to break up any loose chemical combination of sugar with other constituents of the serum, thus yielding values for the sugar which should more nearly approximate to those obtained by us on coagulation. One might anticipate, also, some hydrolysis of any hydrolysible sugars. We have, therefore, here not only determined the sugar value by dialysis after coagulation, but have also added hydrochloric acid to the serum and saline at the beginning of dialysis, and boiled for a few minutes. Before estimating the sugar in this acid saline, it was brought to boiling and neutralised.

The results obtained are given below.

*Experiment VIII.*—Pig's serum dialysed for forty hours against 300 c.c. saline.  
Sugar percentage (mean of two determinations in each case) :—

BY DIALYSIS			BY PRECIPITATION
Uncoag. control	Coagulated	Acid coagulation <sup>1</sup>	With mercuric chloride
0·0807%	0·1318%	0·1635%	0·1191%

*Experiment IX.*—Pig's serum dialysed for forty hours into 300 c.c. saline.  
Percentage of sugar on :—

DIALYSIS AFTER—		PRECIPITATION	
Coagulation	Coag. with HCl <sup>2</sup>	1	2
0·1019%	0·1578%	0·1062%	0·1071%

*Experiment X.*—Pig's serum dialysed for forty-four hours into 300 c.c. saline.  
Percentage of sugar on :—

DIALYSIS OF—		PRECIPITATION	
Control	Coagulated Serum	1	2
0·0942%	0·0981%	0·1098%	0·1106%

(mean of two determinations).

1. Here 2 per cent. of ordinary strong HCl was added to both serum and saline, and the whole boiled for a few minutes at the beginning of dialysis.

2. Here only 1 per cent. of strong HCl was used.

From a comparison of these figures it is difficult to see what the value for the sugar obtained by precipitation of the protein with acid mercuric chloride actually represents. On the one hand in two cases it is greater than that obtained by dialysis after coagulation, while on the other hand it is considerably less than our value after boiling of the serum with dilute acid. The results probably point to a partial hydrolysis of some polysaccharide with, at the same time, a partial destruction of the sugar by the prolonged boiling necessary to remove the last traces of hydrogen sulphide. The very high values obtained by dialysing the serum after first boiling it with hydrochloric acid is another proof of the existence of hydrolysible sugar in the blood, as maintained by Pavy.<sup>1</sup>

### CONCLUSIONS

By this method of estimating the sugar in blood some insight has been gained as to the condition of that sugar. There is evidence to show that part of it exists as free sugar, another part as combined sugar, by this being understood those combinations of glucose with protein or lecithin already referred to, and a third part as polysaccharide or hydrolysible sugar. While it is difficult, even by this method, to discriminate sharply between these various forms of sugar, nevertheless it is a distinct improvement over the mercury precipitation method which yields figures hard to interpret. The method, moreover, is simple and yields concordant results, while it enables one to follow more closely the varying sugar-content of the blood under varying conditions. Although the time interval in making a determination of the sugar by this method is greater than by precipitation methods, yet the actual time involved in carrying out the work of determination is very much less. Furthermore, the work has shown that the corpuscles are devoid of sugar, which is entirely confined to the serum.

1. *Journ. of Physiol.*, XXVI, 282.

## A CASE OF SPECIFIC ADSORPTION OF ENZYMES

By S. G. HEDIN.

*From the Lister Institute of Preventive Medicine**(Received December 31st, 1906)*

In this journal I have recently given an account of experiments on the anti-tryptic action of charcoal.<sup>1</sup> The process by which trypsin was rendered inactive could be divided into two consecutive stages: the mere adsorption or taking up of the trypsin by the charcoal, and the fixation of the same. The amount of trypsin adsorbed could be represented by the difference between the digestive power of the trypsin solution before the treatment with charcoal and that of the filtrate after the treatment. In addition it was found that the action of charcoal was very much like that of the tryptic antibody in the serum, and it was suggested that the effect was brought about in the same way in both cases.

I have extended these investigations, as far as the mere adsorption is concerned, to the proteolytic enzymes of the spleen, at the same time trying the influence of another solid substance, namely, kieselguhr. That kieselguhr may be capable of taking up enzymes, has been shown by Dauwe.<sup>2</sup> Previously I have found that the spleen of the ox contains at least two proteolytic enzymes, the one acting principally in an alkaline medium ( $\alpha$ -protease) and the other in an acid ( $\beta$ -protease).<sup>3</sup> In addition, the spleen (and the serum) contains an antibody which decidedly checks the digestion with alkali, whilst it has practically no effect upon the digestion with acid.<sup>4</sup> Since this antibody is destroyed by the action of weak acetic acid, the spleen substance shows a stronger effect with alkali after previous treatment with acetic acid than it does without treatment with acid. After previous treatment with acid the spleen-pulp gave,

1. *Bio-Chemical Journ.*, I, 484, 1906.

2. *Hofmeisters Beiträge*, Vol. VI, p. 427, 1906.

3. *Journ. of Physiol.*, XXX, p. 155, 1903.

4. Hedin: 'An explanation of the influence of acid and alkali on the autolysis of organs' (*Hammarstens Festschrift*, Upsala, 1906).



roughly, the same effect with alkali as with acid. On trying the effect of juice pressed from spleen substance after grinding with silver sand and mixing with kieselguhr, it was, however, found that even after previous treatment of the juice with acid the effect was much stronger with acid than it was with alkali. This I suggested to be due to the antibody present neutralising the  $\alpha$ -protease more readily in the liquid juice than in the semi-solid spleen pulp. Lately I have found that there is another factor which tends to produce the same result. As shown by the following experiments, kieselguhr adsorbs the  $\alpha$ -protease more readily than it does the  $\beta$ -protease. Since a very large amount of kieselguhr has been used in preparing the spleen-juice, very much of the  $\alpha$ -protease has no doubt been detained in the press-cake, adsorbed by the kieselguhr, whilst the  $\beta$ -protease has passed almost entirely into the filtrate.

A mixture of the two enzymes was prepared by digesting the spleen with 0.1 per cent. acetic acid at  $37^{\circ}$  for two days. Then the fluid was filtered off, and in order to obtain a more concentrated enzyme solution, the filtrate was precipitated by saturation with  $\text{Am}_2\text{SO}_4$ . The salt was removed from the precipitate by dialysis, and the resulting solution was used for digestion.

In order to be able to estimate roughly the amount of enzymes adsorbed, I have tried whether the enzymes, like trypsin, give results proportional to the amounts of enzyme present when digested with a large amount of casein.

200 c.c. of 2.5 per cent. casein in 0.25 per cent.  $\text{Na}_2\text{CO}_3$  solution was taken to each sample. In the case of the  $\beta$ -protease, acid reaction was effected by adding 5 c.c. of 20 per cent. acetic acid to each specimen. The estimation of the digestion which had taken place was carried out by determining the nitrogen in equal volumes of the tannic acid filtrates, as described in previous papers. The figures given represent cubic centimetres of decinormal acid required for neutralising the ammonia obtained.

		With alkali	With acid
10 c.c. Protease sol. + 10 c.c. $\text{H}_2\text{O}$ ...	...	12.2	12.9
20 c.c. „ „ ...	...	28.85	24.1

In accordance with what I have found before,<sup>1</sup> the effect with alkali shows a tendency to increase more rapidly than the amount of enzyme. On the other hand, the effect with acid is very nearly proportional to the amount of enzyme.

The charcoal used was animal charcoal, prepared from bones and purified with HCl. The kieselguhr contained only traces of substances soluble in water and the watery extract of the same gave a perfectly neutral reaction.

In order to effect adsorption of the enzymes equal volumes of enzyme mixtures, prepared as above, were heated with equal weights of charcoal and of kieselguhr *in a neutral medium* at 37° for about two hours. In the same way a control was heated without any adsorbing substance. Then the charcoal and the kieselguhr were filtered off and equal volumes of the control and of the filtrates were taken for digestion with casein, as above.

The results obtained with two different enzyme mixtures were as follows :—

				I		
				With alkali	With acid	Effect with acid
						Effect with alkali
Without adsorption ...	...	...	22.8	31		1.36
After adsorption by charcoal		...	4.2	6.05		1.44
„ „ kieselguhr		...	9.3	27.7		3.0
				II		
Without adsorption ...	...	...	11.5	19.6		1.7
After adsorption by charcoal		...	3.6	6.3		1.75
„ „ kieselguhr		...	6.0	17.7		2.95

Since the same weight of charcoal and of kieselguhr was used, charcoal in this case exercises a more powerful adsorption than does kieselguhr. Both enzymes are adsorbed equally readily by charcoal, the ratio between the effect with acid and with alkali being the same after the adsorption as before.

On the other hand, the effect of the kieselguhr is different inasmuch as a pronounced reduction in the digestion with alkali takes

1. *Hammarstens Festschrift.*

place, whilst the digestion with acid is very little affected. In fact the slight diminution in the digestion with acid might depend upon adsorption of the  $\alpha$ -protease, since this enzyme, although acting principally with alkali, might be able to exercise some slight action with acid too. Kieselguhr therefore evidently adsorbs a considerable amount of  $\alpha$ -protease, whilst it must be left undecided whether a slight adsorption of the  $\beta$ -protease takes place or none at all.

In the above experiments it was not proved that the process of adsorption had come to an end when the casein was added, and therefore the results obtained do not necessarily correspond to the final distribution of the enzymes. The results show that the *rate of adsorption* by charcoal is approximately the same for both enzymes, whilst the *rate of adsorption* by kieselguhr is higher for the  $\alpha$ -protease than for the  $\beta$ -protease.

The experiments bear out the fact that the adsorption of enzymes can be, to a large degree, specific.

In this connection it is interesting to note similar experiments carried out by Bayliss,<sup>1</sup> who found that filter paper adsorbs congo-red and acid fuchsin in the same proportions, like charcoal does the two spleen enzymes, whereas gelatine adsorbs a larger proportion of congo-red than it does of acid fuchsin, thus forming a parallel to the kieselguhr in the case of the spleen enzymes.

The amounts of spleen enzymes adsorbed by charcoal in the above experiments are rather insignificant, as compared with the enormous amounts of trypsin adsorbed by the same amount of charcoal in my investigations on the adsorption of this enzyme.<sup>2</sup> On the whole, charcoal seems to possess quite an exceptional aptitude for adsorbing trypsin. This might, perhaps, depend upon some specific relationship.

It must, however, be borne in mind that the trypsin solutions used in my experiments were nearly free from proteins, whilst the solutions of the spleen enzymes held large amounts of proteins, which could not be digested away lest the enzymes were too much weakened. In the case of trypsin, it was found that the addition of

1. *This Journal*, I, p. 212, 1906.

2. *This Journal*, I, p. 484, 1906.

casein prevents some trypsin from being adsorbed by the charcoal ; in fact, casein is capable of extracting trypsin already adsorbed by the charcoal. Therefore the large amounts of proteins present might account for the spleen enzymes being less readily adsorbed by charcoal than trypsin. On the other hand, the different behaviour of the two spleen enzymes to kieselguhr cannot be accounted for by a different amount of proteins, because they were acted upon in the same fluid.

In conclusion, I wish to call attention to rather an important conclusion which can be drawn from the above experiments, namely, that juices prepared with kieselguhr, as proposed by Buchner in the case of yeast, do not necessarily contain the enzymes in the same proportions as in the cells used. The enzymes might be adsorbed by the kieselguhr, or perhaps by the solid remains of the cells, and consequently some of them might, perhaps, be completely retained in the press-cake.

#### SUMMARY

The  $\alpha$  and  $\beta$ -protease in the spleen of the ox, are adsorbed in the same proportions by charcoal, whilst kieselguhr adsorbs the  $\alpha$ -protease much more readily than the  $\beta$ -protease, and it seems doubtful whether the latter be adsorbed at all. This fact might account for the observation that juice pressed from the spleen after grinding and mixing with kieselguhr contains relatively less  $\alpha$ -protease than does the organ itself.



## THE RELATION BETWEEN THE OSMOTIC STRENGTH OF CELL SAP IN PLANTS AND THEIR PHYSICAL ENVIRONMENT

BY ERIC DRABBLE, D.Sc., F.L.S. AND HILDA DRABBLE.

*(Received January 14th, 1907)*

The literature relating to methods of determining the osmotic strength of the cell sap in plants is voluminous. Foremost amongst writers on the subject have been De Vries (1, 2, 3, 4, 5), Pfeffer (6), Stange (7), Janse (8 and 9); and many other authors have contributed to our knowledge. But in spite of the attention which has been directed to this line of investigation, few authors have given the results of comparative work on a series of plants, though scattered throughout the literature are many isolated instances which, if brought together, would tell us something of the real range of osmotic strength met with. The results obtained seem to show that in general the osmotic strength of sap in land plants ranges between strengths equivalent to 1·3 and 2 per cent. potassium nitrate. It is unfortunate that in the majority of cases the equivalents have been given in percentages of the salt used, for this has greatly obscured the true significance of the results. At the present time it is customary to quote the strengths as equivalent to fractions of gram-molecular solutions; but even stated in this way the results obtained with different electrolytes are not strictly comparable. The best way is to give the osmotic pressures as equal to that of so many millimetres of mercury.

In 1905 a short preliminary statement of the results of our work on the osmotic strengths of sap in plants growing under different

conditions was published (10). In this note determinations for nine plants were described. The number of plants investigated has now been brought up to forty-eight. They have been collected from many very different sources, and the relation between external environment and strength of cell sap has been clearly established.

#### METHOD

The method of plasmolysis employed by de Vries has been used throughout, the plasmolysing agent employed being sodium chloride. Solutions differing by a unit in the second place of decimals were made up from 0.10 to 0.60 gram-molecular strength.

The epidermis, stripped from the surface of the leaf as described by de Vries (3 and 5), is the easiest tissue to work with when it contains a coloured sap as in *Tradescantia* or *Saxifraga sarmentosa*, but in the large majority of cases the epidermis is devoid of such coloured sap, and, moreover, it can only be removed from the leaf with difficulty. On this account the mesophyll has been used in nearly every case, and owing to the presence of chlorophyll corpuscles in these cells, the first appearance of plasmolysis could very certainly be detected after considerable experience of the method. A small portion of the leaf was teased on the slide in water, and was then carefully examined for comparison with the same tissue in the different strengths of solution, commencing with the lowest, 0.10. At a certain concentration, plasmolysis just commences. The strength of solution which just fails to plasmolyse is then taken as the osmotic equivalent of the cell sap. The solutions were allowed to remain on the tissue for from five to ten minutes, and it is important to note that if the cells showed no sign of plasmolysis at the end of this time no plasmolysis could be induced by lengthened exposure to this strength of solution. Hence any objection to the method based on the supposition that treatment with the solutions was not sufficiently long-continued would appear to be without foundation. The mesophyll cells have very thin, readily-permeable cellulose walls, through which the solutions penetrate very rapidly. Penetration through the walls of the epi-

dermal cells is frequently not nearly so rapid, and this is another disadvantage in using the epidermis for making the determinations.

The choice of sodium chloride as the plasmolysing agent is justified from several points of view. In dilute solutions it has only a very slightly toxic action if any, even when exposure to its action is prolonged. It is a salt of which the plant has much experience in nature, sodium chloride being a constituent of cell sap, and also present, often in considerable quantity, in the soil water. Experiment has shown that cells may be plasmolysed with quite strong solutions of this salt, as much as 1-gram-molecular, without losing the power of recovery when again placed in water. Our experience has been in full accord with the statement made by Livingston (11) that sodium chloride penetrates the cells of most plants very slowly if at all, since cells placed in a solution of this salt just strong enough to cause plasmolysis will not recover even after several hours.

True (12) states that a 0.1 gram-molecular solution of sodium chloride will kill *Spirogyra* in twenty-four hours: our own experience, however, does not support this statement. In any case, sodium chloride may be used with confidence as the agent of plasmolysis, especially in view of the fact that the cells are exposed to its action for a short time only.

The osmotic pressures determined by the method thus described can be stated in fractions of a gram-molecular solution of sodium chloride. But this salt is an electrolyte, dissociating in different degree for each stage of dilution. Not only do the undissociated molecules in the solution act osmotically, but the ions play a similar part. A bare statement of osmotic strengths in fractions of a gram-molecular solution does not take into account the fact of ionization, and hence results obtained by using different plasmolysing agents, stated thus, are no more comparable with one another than if they were expressed in percentages.

We have made a calculation of the dissociation-constant  $\alpha = \frac{\lambda_v}{\lambda_\infty}$  for every strength of solution used. From this the osmotic

pressure reckoned in millimetres of mercury can be calculated for any solution. Expressed in this way the results obtained are quite comparable with those obtained by any other method.

The size of the mature mesophyll cells does not diminish sensibly when they are placed in the solutions of sodium chloride. Had there been any great decrease in size it would have been necessary to allow for this in calculating the osmotic strength of sap in the turgid cell, as was pointed out by Jost for merismatic cells from the apices of plants. Free cells such as yeast and *Protococcus* sensibly diminish with increasing strength of the solution, in which they are placed, as will shortly be shown.

### RESULTS

The osmotic pressures determined in the plants examined are expressed in the following table :—

Plant	Situation	Grm. mol. of NaCl	Pressure in mm. mercury at 0°C. & 760 mm.
<i>Callitriche</i> sp. ... ..	Submerged in bog, Cathole, Derbyshire	0·11	3389·60
<i>Myosotis palustris</i> , Relh.	Partially submerged in bog, Cathole, Derbyshire	0·11	3389·60
<i>Nasturtium officinale</i> , R. Br.	Partially submerged in bog, Cathole, Derbyshire	0·11	3389·60
<i>Ranunculus repens</i> , L. ...	On saturated mossy tufts in bog, Cathole, Derbyshire	0·11	3389·60
<i>Taraxacum officinale</i> , Web.	Damp grass near ditch by Thames, Kew	0·11	3389·60
<i>Angelica sylvestris</i> , L. ...	On mossy tufts in bog, Cathole, Derbyshire	0·12	3728·56
<i>Taraxacum officinale</i> , Web.	Garden, Upper Brighton, Cheshire...	0·12	3728·56
<i>Hydrocotyle vulgaris</i> , L. .	Dryer part of bog, Cathole, Derbyshire	0·13	4067·52
<i>Cnicus palustris</i> , Willd.	Dryer part of bog, Cathole, Derbyshire	0·13	4067·52
<i>Prunella vulgaris</i> , L. ...	Dryer part of bog, Cathole, Derbyshire	0·13	4067·52
<i>Spiraea</i> sp. ... ..	Shrubbery on sandy soil, Sunningdale	0·13	4067·52
<i>Mahonia</i> sp. ... ..	Shrubbery on sandy soil, Sunningdale	0·13	4067·52
<i>Saxifraga sarmentosa</i> , L.	Cool-house, Chelsea Physic Gardens	0·13	4067·52
<i>Brassica oleracea</i> , L.(Cabbage)	Garden, Upper Brighton, Cheshire...	0·13	4067·52
<i>Athyrium Filix-foemina</i> , Roth.	Exposed wood on Bidston Hill, Cheshire, overlooking sea	0·14	4406·48
<i>Taraxacum officinale</i> , Web.	Exposed wood on Bidston Hill, Cheshire, overlooking sea	0·15	4406·48



Plant	Situation	Grm. mol. of NaCl	Pressure in mm. mercury at 6° C. & 760 mm.
<i>Oxalis acetosa</i> , L. ...	Exposed wood on Bidston Hill, Cheshire, overlooking sea	0·17	5253·88
<i>Rubus fruticosus</i> , L. ...	Exposed wood on Bidston Hill, Cheshire, overlooking sea	0·17	5253·88
<i>Zygnuma</i> sp. ...	Submerged in brackish ditch, Leasowe, Cheshire	0·17	5253·88
<i>Geranium Robertianum</i> , L.	Near, but not in, salt marsh, North- fleet	0·17	5253·88
<i>Chenopodium urbicum</i> , L.	Sand in garden, Upper Brighton, Cheshire	0·18	5541·99
<i>Stellaria media</i> , Cyr. ...	Sand in garden, Upper Brighton, Cheshire	0·19	5830·11
<i>Rumex crispus</i> , L. ...	Holymoore, Derbyshire ...	0·21	6440·24
<i>Glaucium flavum</i> , Crantz	Near salt marsh, Burton Point, Cheshire	0·22	6745·30
<i>Geranium Robertianum</i> , L.	Damp rocks below Cwm Idwal ...	0·23	7033·42
<i>Rubus fruticosus</i> , L. ...	Sandhills, Wallasey, Cheshire ...	0·24	7287·64
<i>Senecio Jacobaea</i> , L. ...	Sandhills, Wallasey, Cheshire ...	0·24	7287·64
<i>Cotula coronopifolia</i> , L.	Partially submerged in brackish ditch, Leasowe, Cheshire	0·25	7626·64
<i>Rubus fruticosus</i> , L. ...	Holymoore, Derbyshire ...	0·25	7626·64
<i>Pteris aquilina</i> , L. ...	Holymoore, Derbyshire ...	0·25	7626·64
<i>Cnicus lanceolatus</i> , Willd.	Holymoore, Derbyshire ...	0·25	7626·64
<i>Sedum acre</i> , L. ...	Sandhills, Wallasey, Cheshire ...	0·26	7880·82
<i>Calluna Erica</i> , DC. ...	Holymoore, Derbyshire ...	0·26	7880·82
<i>Galium saxatile</i> , L. ...	Holymoore, Derbyshire ...	0·26	7880·82
<i>Uaccinium Myrtillus</i> , L.	Holymoore, Derbyshire ...	0·27	7916·40
<i>Hieracium</i> sp. ...	Rocks above Cwm Idwal ...	0·28	8474·00
<i>Uaccinium Myrtillus</i> , L.	Rocks above Cwm Idwal ...	0·28	8474·00
<i>Taraxacum officinale</i> , Web.	Sandhills, Wallasey, Cheshire ...	0·28	8474·00
<i>Hypochaeris radicata</i> , L.	Sandhills, Wallasey, Cheshire ...	0·28	8474·00
<i>Potentilla reptans</i> , L. ...	Bare burnt patch, Holymoore, Derbyshire	0·28	8474·00
<i>Glaux maritima</i> , L. ...	Edge brackish ditch, Leasowe, Cheshire	0·29	8812·96
<i>Brassica monensis</i> , Huds.	Sandhills, Wallasey, Cheshire ...	0·29	8812·96
<i>Anthyllis Vulneraria</i> , L....	Sandhills, Wallasey, Cheshire ...	0·29	8812·96
<i>Plantago lanceolata</i> , L. ...	Sandhills, Wallasey, Cheshire ...	0·29	8812·96
<i>Galium verum</i> , L. ...	Sandhills, Wallasey, Cheshire ...	0·29	8812·96
<i>Festuca ovina</i> , L. ...	Salt marsh, Burton Point, Cheshire...	0·50	14660·02
<i>Aster Tripolium</i> , L. ...	Salt marsh, Burton Point, Cheshire...	0·51	14958·13
<i>Plantago maritima</i> , L. ...	Salt marsh, Burton Point, Cheshire...	0·51	14958·13

A point which has come out very clearly in the results summarised in the preceding table is the striking relation between external conditions and the strength of cell sap. In all cases plants growing in situations in which they are subjected to physiological drought are provided with cell sap of high osmotic strength. If the physiological drought had been accompanied by a diminution in turgescence of the cells, the increased osmotic strength might conceivably have been due to abstraction of water and a resulting concentration of salts in the remaining portion. This, however, is not at all the state of affairs, for the plants examined were in all cases completely turgid. Hence it is the cell sap, with its normal complement of water, which is osmotically strong. The inter-dependence of osmotic strength and environment may be illustrated by considering in detail some of the results tabulated in the list just given.

*Bog plants.*—The bog plants examined were collected in a bog at Cathole, in Derbyshire, situated on the shaly clay immediately underlying the uppermost grit of the Millstone series. The bog consists of small pools, amongst which are tufts of moss, principally *Sphagnum* and *Hypnum*, which are saturated with water. In the pools grow *Callitriche* sp., *Myosotis palustris*, Relh., and *Nasturtium officinale*, R.Br. The submerged *Callitriche* is in contact with an unfailing supply of pure water; its osmotic strength is 3389·60 mm. The partially submerged plants, *Myosotis* and *Nasturtium* are in closely similar circumstances with regard to their water supply, and their sap has the same strength as the wholly submerged plants. In the partially submerged plants it is true a fairly rapid transpiration may take place, but the supply which compensates for this is readily accessible at all times. *Ranunculus repens*, L., growing on the saturated tufts of moss is in similar circumstances, and has sap of the same strength. All these are low-growing plants, and are not much exposed to the wind. *Angelica sylvestris*, L., grows in the same places as *Ranunculus repens*, but it is a tall plant and is much more exposed to the wind, which will promote transpiration. Its strength of sap is greater—3728·56 mm. The low-growing plants are not only protected from the wind by the surrounding vegetation, but, as Schimper

(15) has mentioned, the actual velocity of the wind is very greatly retarded as the surface of the ground is approached. The plants growing on the stiff clay at the edge of the bog have an increased strength of cell-sap—4067·52 mm. Here the water supply, though adequate, is not so immediately accessible as in the former cases. These plants are *Hydrocotyle vulgaris*, L., *Cnicus palustris*, Willd., and *Prunella vulgaris*, L.

By the side of the Thames at Kew, growing amongst long grass in a very damp situation, was gathered a large specimen of *Taraxacum officinale*, Web. This agreed in its strength of sap with the lowest of the Cathole bog plants—3389·60.

*Garden Plants.*—From a garden at Upper Brighton, Cheshire, on a sandy soil, *Taraxacum officinale*, Web., was growing on a humus surface covered by a dense growth of weeds. It is well known that humus adds considerably to the water-holding capacity of soils (13, 14), and hence in the case under consideration a plentiful supply is ensured. The osmotic strength was found to be 3728·56 mm. A cabbage growing in a cleared portion of the same soil had a less efficient water-holding substratum, and its osmotic strength was 4067·52.

Sir Joseph Hooker's garden at Sunningdale lies on a very sandy soil, with a consequently low water-holding capacity. This has been largely counteracted by the addition of a large quantity of humus. *Spiraeae* sp. and *Mabonia* sp., gathered in the shrubbery, showed a strength of sap equal to 4067·52 mm. of mercury. This value is greater than that found in garden plants from a clay soil, when the strength is generally 3728·56.

*Wood plants from an exposed wood.*—Bidston Woods lie on a hill overlooking the sea and are greatly subject to high winds. The cell sap of the plants growing there varies from 4406·48 in a low-growing plant like *Athyrium Filix-foemina*, Roth., surrounded by other vegetation and generally confined to sheltered hollows, through *Taraxacum officinale*, Web., 4626·80 mm., found in more exposed places, to *Rubus fruticosus*, L., 5253·88 mm., growing well above

the surrounding undergrowth and hence more exposed to the wind. The sap of *Oxalis acetosa*, L., with its high oxalic acid content, has a strength equal to that of *Rubus*, in spite of its low-growing habit. This is a point to which reference will be made below.

*Moorland plants.*—These were collected from Holymoore in Derbyshire, where extensive tracts of moorland lie on the dip-slope of the first Millstone Grit, at an elevation of about 1,000 ft. At the edge of the moor there is a fairly good soil, where grass and other plants flourish. *Rumex crispus*, L., gathered here, has a sap strength of 6440·24 mm. An increase of strength was at once found in the plants which penetrate on to the moorland proper. Here the soil is dry and the whole area is subject to high winds. In accordance with the physiological dryness of the environment the strength of sap is 7626·64 mm. This is found in *Pteris aquilina*, L., *Rubus fruticosus*, L., and *Cnicus lanceolatus*, Willd.

Passing into the midst of the heather moors the principal plants are *Calluna Erica*, DC., and *Galium saxatile*, L., with a strength 7880·82 mm., and *Vaccinium Myrtillus*, L., with 7916·40 mm. The greater strength of the sap in this last plant will be referred to again below.

It is customary to set fire to the heather in certain parts of the moor each year. These burnt patches thus become possessed of a dry soil, rich in salts. Colonisation of these burnt patches takes place slowly. The first plant to establish itself is *Potentilla reptans*, L., which only attains a small stunted, woody habit, and possesses a strong sap, with pressure equal to 8474·80 mm. of mercury.

*Mountain plants.*—These were collected on the hills above Cwm Idwal, in North Wales. The conditions vary considerably from place to place. The rocks near the streams are comparatively damp, and on these grow many plants which are not typically mountain forms, such as *Geranium Robertianum*, L. This plant had a strength of sap equal to 7033·42 mm. of mercury. The plants growing on the nearly bare rocks of the exposed summits of the hills showed a greatly increased strength of sap—8474·00 mm.



*Sand-dune plants.*—These were collected from the sand dunes of the Wirral Peninsula. Here the conditions are conducive to extreme xerophily. The sand in many places is almost without admixture, and its water-holding power is very small (14). The high sea winds, the absence of any shade from the direct rays of the sun, and the salt spray which at the highest tides is blown over the dunes, all tend to render the conditions extremely dry physiologically.

Growing on the barest patches of the sand, in addition to *Psamma arenaria*, R. and S., are *Brassica monensis*, Huds., *Anthyllis Vulneraria*, L., *Plantago lanceolata*, L., and *Galium verum*, L., all with the high strength of sap—8812·96 mm. When decaying vegetation has added humus to the sand other plants obtain a hold, amongst which are *Hypochaeris radicata*, L., and *Taraxacum officinale*, Web., with a strength of sap—8474·00. Both these plants have long tap-roots, from 18 inches to 3 feet in length, when growing in the sand. At the same time their evaporating surface is comparatively small, and hence they can economise their water supply. *Senecio Jacobaea*, L., is still better adapted to life in these regions. Its tap-root is long and branched, and in the water-holding humus layer lying on the surface of the sand it produces a dense growth of lateral roots. Having thus a good command of the water supply, its osmotic strength, 7287·64, is lower than in other plants in the same situation less well adapted. *Sedum acre*, L., with a comparatively small root system, and hence a limited area from which to draw its supply of water, has a high osmotic strength of 7880·82 mm.

*Brackish water plants.*—These were collected from ditches inside the Leasowe embankment in the Wirral Peninsula. Three plants were selected for examination. Unfortunately the results are not quite comparable, as the plants were not all collected from the same ditch, and the concentration of salt in the water varies slightly from ditch to ditch. In spite of this the results are striking. The submerged *Zygnema* has a strength of 5253·88. There can be no lack of water, and the high osmotic strength serves principally to resist the physical action of the brackish water.

In some of the ditches, *Cotula coronopifolia*, L., an alien plant introduced some years ago, has spread rapidly. It grows rooting in the water, but with its leaves exposed to the air, with resulting transpiration. Its strength of sap is higher than in the submerged alga, being 7626·64 mm. *Glaux maritima*, L., on the edge of the ditch and liable to be reached by the water occasionally, though usually raised above it, has a strength of 8812·96 mm. In this last case, when the water-level falls, some water will be left in the soil, and, on evaporation, will add to the percentage of salts present in the water available to the plant.

*Salt-marsh plants.*—These were gathered on the Burton Point marshes, by the side of the Dee. The level expanse of marsh is covered at each recurrence of high tide. Here grow *Aster Tripolium*, L., and *Plantago maritima*, L., with sap strength 14958·13 mm. of mercury, and a variety of *Festuca ovina*, L., with strength 14660·02 mm. The cells of the plants on this moist soil would be plasmolysed twice a day, if they did not possess a high osmotic strength of sap which offers considerable resistance to the water-extracting action of the sea. When the tide recedes the plants are exposed to a progressively concentrating soil-water, which must render physiological drought a very potent factor in the plant's daily life.

A further point calls for notice. In the garden at Upper Brighton is a sandy patch, with very little admixture of humus; the situation is, however, sheltered from direct insolation and from high winds. Here the chief plants are *Chenopodium urbicum*, L., and *Stellaria media*, Cyr., growing under almost precisely similar conditions. *Chenopodium* has a greater height and is hence more exposed to the action of the wind. One might reasonably expect that the osmotic strength of the sap would be higher than in *Stellaria media*, but the reverse is the case. Again, on the moors, *Calluna Erica*, DC., and *Vaccinium Myrtillus*, L., have a close similarity of growth, and yet the cell-sap of *Vaccinium* is stronger than that of *Calluna*. In Bidston Woods, the low-growing *Oxalis* has a greater strength of sap than *Taraxacum* or *Athyrium*, and equal to the much exposed *Rubus*.

A striking feature in the plants which have thus shown a departure from the expected strength of sap is the thin and sometimes delicate texture of their leaves and the small development of cuticle, as compared with the leaves of their neighbours. Structurally they are more subject to loss of water; as will be mentioned below, this tendency is, to some extent, compensated by the increased concentration of the cell sap.

It appears from a general review of the results obtained, that for any given area, the plants which have most difficulty in obtaining an adequate supply of water have a higher concentration of cell sap than have their more favourably situated neighbours. Also, the osmotic strength of plants as a whole growing in any district is generally influenced by the physiological drought of the district.

#### PHYSIOLOGICAL BEARING OF RESULTS

##### (i) *Effect of increased concentration of sap on Transpiration.*

Increase in concentration of a solution lowers the vapour pressure of the solvent. Thus, the addition of a salt to water lowers its vapour tension proportionally to the amount of the salt added.

The dependence of vapour tension on osmotic strength has been calculated, and is found to be expressed by the following formula :—

$$P = \frac{\pi - \pi_1}{\pi} \cdot \frac{0.0819T \times 1000S \times 760}{M}$$

When P is the osmotic pressure at absolute temperature T expressed in millimetres of mercury,  $\pi$  and  $\pi_1$ , are the vapour tensions of the solvent and solution at the temperature T, S is the specific gravity of the solution, and M the molecular weight of the pure solvent.

Hence we can find the vapour tension of any solution whose osmotic pressure is known.  $\pi$  can be found from the published tables, T is the observed temperature, S in all cases dealt with in our work is sensibly equal to unity and 1000 S becomes 1000, and M is 18.

Now the vapour tension is directly proportional to the rate of evaporation from a free surface of the solution, and thus it is possible to calculate the relative rate of evaporation from any two solutions of known osmotic strength. Substituting in the above equation the values for the experimental conditions we have for 18° C. :—

$$P = \frac{15.4 - \pi_1}{15.4} \cdot \frac{0.0819 (273 + 18) \times 1000 \times 760}{18} \\ \left(1 - \frac{\pi_1}{15.4}\right) \times 1006278$$

Thus for the osmotic pressures 3728 mm., 8812 mm., and 14958 mm., we obtain the vapour tensions 15.34, 15.26, and 15.17 mm. That is to say, the rates of free evaporation from cell saps of the above osmotic strengths will be proportional to 15.34, 15.26 and 15.17, or as 1.011 : 1.005 : 1. Hence the differences of concentration met with in the cell sap in the plants can exercise but little effect in checking evaporation, and by far the greatest part in conservation of water must be played by structural configuration. The above calculation only applies to free evaporation. How this may be modified by the intervention of a semi-permeable membrane is not known. An attempt is being made to attack the problem, but the experimental difficulties are great.

(ii) *Effect of Concentration of Cell Sap on the Freezing Point.*

One gram molecule of a non-electrolyte dissolved in one litre of water exercising an osmotic pressure of 16948 mm. of mercury will depress the freezing point of a solution by about 1.89° C. Of course decrease in temperature will diminish the ionization in the sap, but as we do not know with precision the true constitution of the sap, this effect, which will be very slight, must be neglected. Let us consider an osmotic pressure of 3728 mm. at 18° C. This, at 0° C., becomes 3467.04 mm. This pressure will depress the freezing point by 0.41° C. In a salt-marsh plant the pressure 14958 mm. at 18° C. becomes 13911 mm. at 0° C., and this will depress the freezing point 1.55° C. Hence increased osmotic pressure may serve to check



freezing of the sap, but in all cases this check must be comparatively slight. The calculations given above are not strictly accurate, but for our purposes they are quite sufficiently so.

(iii) *Effect of High Temperatures on the Osmotic Strength of the Cell Sap.*

A rise in temperature will cause an increase in the osmotic strength of the sap. A rise of  $10^{\circ}$  C. will increase the osmotic pressure of sap in a sand-dune plant from 8813 to 9116 mm. As will be shown below, this will add to the plant's power of absorbing water, and thus to some extent counterbalance the increased loss of water by transpiration due to the higher temperature. The osmotic strength will also be augmented by increased ionization at high temperatures. It is impossible to calculate this as we do not know the composition of the sap. Probably many of the osmotically active substances in the cell are non-electrolytes which will not ionize at all, while the electrolytes are a very heterogeneous mixture, each salt dissociating differently. In the present state of our knowledge it is hopeless to attempt any calculation of ionization and temperature in the cell sap.

(iv) *Effect of Increased Osmotic Strength on the Absorption of Water.*

We have experimented with a number of plants growing under different conditions. In every case we have found them to possess the power of absorbing water more or less rapidly through their leaves. In some cases the structural character of the leaves in the more xerophyllous conditions favours absorption. Thus the dandelion from the garden has a smooth epidermis which neither holds nor absorbs water readily, while the same species from the sandhills has a rough hairy surface for holding and absorbing moisture. It seems probable that many of the plants growing under conditions of physiological drought normally obtain a considerable quantity of water by direct absorption of precipitated atmospheric moisture through their leaves.

The rate of absorption of water through the protoplasmic layer,

which for our purposes is considered as semi-permeable, will be proportional to the sucking force, namely the osmotic strength of the cell sap. Now we have seen that a great range of osmotic strength is met with in saps. The rate of absorption will be proportional to these strengths of sap, leaving out of account the structural impediments. Thus, neglecting the last-named factor, and considering a garden plant with osmotic strength of sap 3728, a sand-dune plant with 8813, and a salt-marsh plant with 14958, the rate of absorption of water through these leaves will be in the ratio of 1.00 : 2.36 : 4.01. That is to say, a sand-dune plant in virtue of its strength of sap, and neglecting structural modifications, will suck up water more than twice as quickly, and a salt-marsh plant four times as quickly as an ordinary garden plant. To some extent also the rate of absorption of soil moisture through the roots must be proportional to the strength of sap.

It is perfectly evident from a consideration of the environment of the different plants examined that some are much more liable to dessication than others. The submerged plant has an abundance of water at all times, and apparently all that is necessary is for the sap to have a concentration slightly greater than that of the external medium. In a land plant, however, conditions of dessication may set in, and on the sandhills this is very liable to occur. Hence, if a plant can rapidly absorb through its leaves the water of transient showers it will reap an immediate benefit, since such small quantities of rain are unable to penetrate to the roots of the plant. Hence there appears to be a very considerable value attached to concentration of cell sap, quite apart from its importance in the function of root absorption.

In plants liable to be submerged by salt water the value of a rapid absorption of rain water is obvious, though probably the primary utility of the high strength in this case is resistance to the water-extracting action of the sea.

## SUMMARY

1. The osmotic pressure of the sap in the turgid cell in the plants examined varies between 3389·60 mm. and 14958·13 mm. of mercury.
2. The osmotic strength is least in submerged freshwater plants and greatest in salt-marsh plants.
3. The greater the physiological drought under which the plants are accustomed to grow, the greater the osmotic strength of the sap in the turgid cell.
4. In any area the osmotic strength varies with the physiological scarcity of water.
5. In all plants growing under the same conditions the osmotic strength of the cell sap is generally the same.
6. Where two plants, growing under similar conditions, have a marked difference in the structural arrangements for checking loss of water by transpiration, that plant with the less adequate anatomical provision has the greater strength of cell sap.
7. The *direct* effect on transpiration of increased osmotic strength of sap will be negligible.
8. The depression of the freezing point of the sap within the range of strengths found will be small, but may be effective in the case of plants with the highest osmotic strength of sap.
9. The effect of increased osmotic strength of sap on absorption will be marked, the rate of absorption of water being proportional to the osmotic strength of the sap.
10. The effect of increased temperature on the osmotic strength of sap will be quite appreciable and will tend to enhance the power of absorption of water by the plants.

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## ON THE CATALYTIC DECOMPOSITION OF HYDROGEN PEROXIDE BY THE CATALASE OF BLOOD

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Schönbein (1) in 1856 called attention to the fact that when extracts of many animal and vegetable tissues were treated with tincture of guaiacum in presence of air, a blue colour was developed. He also showed that the extracts possessed the power of decomposing hydrogen peroxide into water and free oxygen, and believed that both these properties were common to all ferments. Schmidt (2), 1872, suggested that in the case of blood these two reactions were both due to haemoglobin, and not to the presence of ferments at all.

Jacobson (3), 1892, demonstrated that when the juices of various plants were carefully heated, the power of breaking up hydrogen peroxide very soon vanished, but that the diastatic properties were retained until a much higher temperature had been reached.

Lépine (4), 1899, suggested that the decomposition of hydrogen peroxide was due to the presence of a definite ferment.

Loew (5), 1900, as the result of extensive investigations, was also led to regard this property as the specific function of a distinct enzyme, to which he gave the appropriate name of *Catalase*. He classed catalase with the oxydases for various reasons, and distinguished two varieties of catalase, the  $\beta$  variety being more readily soluble than the  $\alpha$  form.

Many views have been expressed by different writers as to the possible rôle of catalase and its near relatives the oxygenases and peroxydases in the living organism, but this question has not yet received a satisfactory explanation.

Pozzi-Escot (6), 1902, indicated the probable identity of Loew's Catalase with Rey-Pailhade's 'Philothion,' and was the first to study the velocity of the reaction between solutions of blood and hydrogen peroxide. His conclusions were that the amounts of oxygen liberated were proportional to the amounts of enzyme present, and that the velocity of the reaction was a function of the 'active mass.' The same author also studied the effects of some of the substances that exercise a 'poisonous' influence upon catalase. Ville and Moitessier (7) also enquired into the rate at which the decomposition proceeds; their conclusions were, broadly speaking, in agreement with those of Pozzi-Escot, but were not nearly so explicit.

G. Senter (8), 1903, introduced a useful method for isolating the enzyme in a fairly pure condition in solution from defibrinated ox blood. He called the enzyme *Haemase*, and found that within certain limits a very close approximation to the formula for a reaction of the first order was obtainable when solutions of haemase acted upon dilute solutions of hydrogen peroxide. The effects of various poisons were studied, and the mechanism of their action discussed. Senter and also Neumann Wender (9) showed that the catalytic phenomenon is a function quite distinct from any other enzyme action.

Bach (10), 1905, in the course of investigations on catalase arrived at the same conclusions as Senter and others with regard to the speed of the decomposition, and proved that the times taken by various amounts of catalase to decompose a given mass of  $\text{H}_2\text{O}_2$  decreased much more rapidly than the amount of enzyme increased.

In the same year Senter (11) published an account of further experiments on the effects of various types of retarding substances on the reaction, and put forth some interesting speculations as to their mode of action upon haemase as compared with inorganic catalysts.

Hans Euler (12), working with the catalase of *Boletus scaber*, found that within certain limits the velocity of the action of this enzyme on hydrogen peroxide could be expressed by the usual formula for a reaction of the first order.

Quite recently van Laer (13) has studied the catalyses effected

by ground malt and by yeast, and has shown that there exist optimum concentrations of  $\text{H}_2\text{O}_2$ , at which each material is most active in causing evolution of oxygen. He employed an ingenious graphic method for eliminating from the results the loss of catalase which accompanies the progress of the reaction, and is of the opinion that when this correction is made, the law of mass action for a unimolecular reaction is very closely followed.

The purpose of the present investigation is to re-examine the reaction between solutions of catalase and hydrogen peroxide, in the light of recent conceptions of the nature of enzyme action.<sup>1</sup> It has been clearly shown that on theoretical grounds, a close agreement with the usual formula expressing a reaction of the first order is, where enzyme action is concerned, only possible under certain conditions. The amount of enzyme, for example, should be fairly constant throughout the observation, and the substrate must be dilute.

Moreover, the ratio of enzyme to substrate must not be very small. Even under the very best circumstances the constants at the beginning and end of the reaction frequently deviate a good deal from the mean values. The experiments of A. J. Brown (14), Armstrong (15), and others upon various enzyme actions clearly demonstrate that the laws worked out for inorganic catalysts must be applied with caution to enzymes.

Much uncertainty also exists as to the relation between the concentration and activity of catalase solutions; in fact, nothing very definite seems to have been done in the direction of ascertaining what law this enzyme follows, except within certain narrow limits of concentration.

Preliminary experiments having shown that the reaction was exceedingly susceptible to modification by alterations in the concentration of the hydrogen peroxide, it was thought that by taking this property of catalase into account, some light might possibly be thrown on the questions of the rate of the reaction and relative activity of the enzyme under various conditions.

1. For an excellent summary of modern views of enzyme action see the article on that subject by Prof. Benjamin Moore in 'Recent Advances in Physiology and Bio-Chemistry,' 1926; pub. Ed. Arnold. 18s. net.

## METHODS

Many experimental difficulties are encountered in working with animal catalase. For example, when the ratio of enzyme to substrate is small and the concentration of the latter high, there is a rapid cessation of the action. This appears to be due to a poisonous effect of the hydrogen peroxide on the catalase; it can be to a great extent avoided by working at as low a temperature as is convenient ( $10^{\circ}$  C. or under).

The reactions were, therefore, carried out in a thermostat; in some cases the vessels were surrounded with finely broken melting ice instead. The solutions employed were in all cases brought to the required temperature by a prolonged immersion in the thermostat, or in the broken ice.

The hydrogen peroxide solutions were prepared by suitable dilutions of Merck's Perhydrol (30 per cent.) with pure distilled water free from  $\text{CO}_2$ .

For the enzyme solution, blood drawn direct from the finger, or freshly defibrinated ox blood were sometimes made use of; the best, however, was found to be the 'Haemase' solution prepared from ox blood according to Senter's method (8). This is free from haemoglobin, and seems to contain no other enzymes beside catalase; it keeps well if stored in an ice-chest.

Two methods were employed in order to determine the amounts of peroxide catalysed in given periods of time. The first method consisted in the measurement of the volume of oxygen evolved from the reaction mixture, this volume being subsequently corrected for temperature, pressure and tension of aqueous vapour. This method is the one which has been most extensively used for determining the effect of various catalysors on hydrogen peroxide.

In the other method which is due to Senter (8, 11), the progressive diminution in the concentration of the substrate was directly measured. Known volumes of the reaction mixture were pipetted off at stated intervals of time, and run into dilute sulphuric acid. The acid instantly stopped the action of the enzyme, and the fluids could



then be titrated with a standard solution of potassium permanganate of appropriate strength.

The titration method is best used with Senter's 'Haemase' as the source of the enzyme, since the amount of extraneous organic matter in that solution is so small that it is almost negligible: a control titration is nevertheless necessary in order to determine the volume of permanganate solution required to strike a permanent standard pink with a known volume of a blank mixture. This mixture is made by taking water and enzyme in the same proportions as the peroxide and haemase were used in the reaction mixture.

In the experiments on reaction velocity the values of the constant  $K = \frac{1}{\theta} \log \frac{a}{a-x}$  were calculated in order to ascertain the extent to which the reaction agreed with formula for a monomolecular reaction. (In this formula  $K = 0.4343 k$ .)

Senter (8) has employed a somewhat different formula in his calculations; it has the disadvantage of multiplying experimental errors greatly, and does not, moreover, give very different results to the formula generally used. The usual formula has therefore been retained.

In using the first method of measurement a very serious source of error and inconstancy is soon encountered. The reaction mixture becomes supersaturated with oxygen, which is suddenly liberated on the slightest commotion being set up in the liquid.

The following experiment shows the influence of such disturbances on the volumes of oxygen evolved and on the values of  $K$  :—

TABLE I

60 c.c. of  $H_2O_2$  (= 50 c.c. available  $O_2$ ) + 5 c.c. of enzyme solution. Temperature  $4^\circ C$ .

$\theta$ min.		C.c.'s of oxygen evolved		$K$
1	...	9.8	...	0.0947
2	...	5.2	...	0.0774
3*	...	7.0	...	0.0839
4	...	3.4	...	0.0770
5	...	2.8	...	0.0721
6	...	2.8	...	0.0700
7*	...	4.4	...	0.0760
8	...	1.2	...	0.0715
9	...	1.0	...	0.0673
10	...	0.9	...	0.0639

The contents of the flask were shaken at the commencement of the periods marked by asterisks, i.e., at the beginning of the third and seventh minutes. The increase in the volume of oxygen evolved is well seen.

The process of saturation of the mixture with oxygen is, however, by no means so rapid as might be imagined. Hence the volumes of gas evolved in equal intervals of time during the early stages of the reaction increase until a maximum volume is attained. This point is best brought out when moderate concentrations of  $\text{H}_2\text{O}_2$  are employed; the table given below is a good example of the phenomenon.

TABLE II

200 c.c. $\frac{\text{N}}{5}$ $\text{H}_2\text{O}_2$ + 10 c.c. dilute enzyme solution. Temperature $4^\circ \text{C}$ .		
$\theta$ min.	C.c.'s of oxygen evolved	
3	...	7.95
6	...	12.85
9	...	19.9
12	...	16.3

The time necessary for the tension of the oxygen in the solution to reach its maximum is in this case between nine and twelve minutes.

There can be no doubt that by the use of appropriate stirring gear these physical defects might be greatly reduced; at the same time it is questionable whether the supersaturation error could ever be entirely removed in this way.

Notwithstanding these somewhat serious fallacies the method was frequently made use of since there are details in its favour.

There do not seem to be any marked sources of error attending the use of the titration method when the experiment is properly carried out.

#### INFLUENCE OF VARYING CONCENTRATIONS OF HYDROGEN PEROXIDE

When, in a series of estimations, the relative amount of catalysis effected by equal amounts of enzyme solutions of the same strength acting upon equal volumes of hydrogen peroxide of various concentrations are studied, it is found that the rate of decomposition increases

with increasing concentration of  $\text{H}_2\text{O}_2$  up to a certain point beyond which it again falls off rapidly.

In the experiment given below the rate of catalysis was measured by the collection method, the volume of oxygen being measured in each observation at the end of five, ten and fifteen minutes. The concentration of the peroxide ranged from 30 per cent. to 0.04 per cent. (by weight).

TABLE III

10 c.c.  $\text{H}_2\text{O}_2$  + 1 c.c. strong enzyme. Temperature  $15^\circ \text{C}$ .

	Time	Concentration of hydrogen peroxide					
		30 %	10 %	5 %	2 %	0.4 %	0.04 %
C.c.'s of oxygen	5 minutes .....	1.6	7.6	8.0	22.2	22.3	1.2
	5-10 „ .....	—	1.8	2.6	6.8	3.3	—
	10-15 „ .....	—	2.6	3.4	8.8	0.8	—
	Total 15 minutes ...	1.6	12.0	14.0	37.8	26.4	1.2

There is very little difference between the volumes of gas evolved from 30 per cent. and 0.04 per cent. solutions. The maximum activity was somewhere between 0.4 per cent. and 2.0 per cent.

It is interesting to notice that at several of the concentrations the oxygen liberated during the third period of five minutes is considerably greater than that evolved during the second interval. This phenomenon is always more or less marked when the same method is employed, though the difference may not always be obvious when periods as long as five minutes are under observation: it appears to be due to the fact that the mixture slowly becomes supersaturated with oxygen. During the first five minutes there is a considerable bulk of oxygen evolved; the commotion caused by its brisk liberation is, however, so considerable that very little supersaturation is likely to occur until after the effervescence has in some degree subsided. The reaction mixture would then slowly supersaturate with oxygen, as in the experiment of Table No. II, with the result that the volume of gas evolved during the second interval would be less than that given off during the third.

That this explanation is at least partly correct may be shown by comparing the volume of oxygen evolved with the volume actually resulting from the decomposition as measured by the titration method.

The following table gives the results of two parallel experiments by the two methods :—

TABLE IV

C.c.'s of oxygen at N.T.P. from 5 c.c.  $\text{H}_2\text{O}_2$  and  $\frac{1}{2}$  c.c. enzyme solution  
Temperature  $10^\circ \text{C}$ .

	$\text{H}_2\text{O}_2$ at 2.68 %		$\text{H}_2\text{O}_2$ at 5.72 %	
	Evolved and measured	Found by titration	Evolved and measured	Found by titration
5 minutes .....	11.90	29.00	6.05	14.30
5-10 „ .....	5.97	11.50	3.48	3.47
10-15 „ .....	8.72	3.07	4.95	1.18
	<hr/> 26.59	<hr/> 43.57	<hr/> 14.48	<hr/> 18.95

The amounts of oxygen formed by the decomposition are seen to progressively diminish as the reaction proceeds, whilst those evolved and collected show the variation referred to above. In the case of the weaker hydrogen peroxide the amount of oxygen still retained in the fluid is 16.98 c.c., and in the 5.72 per cent. peroxide 4.47 c.c.

The question that naturally suggested itself after the foregoing experiments was whether the optimum point in the concentration of the  $\text{H}_2\text{O}_2$  varied with the strength of the catalase solution, and if so, to what extent.

In order to decide this point, solutions of laked blood of various dilutions were employed, and the maximum found for each concentration used. The results of a preliminary experiment are given below :—

TABLE V

5 c.c.  $\text{H}_2\text{O}_2$  solution + 0.5 c.c. diluted blood

		$\text{H}_2\text{O}_2$					
		5 %	4 %	3 %	2 %	1 %	0.5 %
C.c.'s of oxygen	5 minutes ...	7.2	9.1	20.7	21.2	19.0	8.9
	5-10 „ ...	3.2	7.1	7.9	13.6	1.2	1.4
	10-15 „ ...	4.3	7.6	7.3	5.4	0.4	0.6
	Total .....	<hr/> 14.7	<hr/> 23.8	<hr/> 35.9	<hr/> 40.2	<hr/> 20.6	<hr/> 10.9

The maximum liberation of gas occurs when the percentage of  $\text{H}_2\text{O}_2$  lies between 2 per cent. and 3 per cent.



TABLE Va

5 c.c.  $\text{H}_2\text{O}_2$  solution + 0.5 c.c. diluted blood (half strength of above)

		$\text{H}_2\text{O}_2$					
		5 %	4 %	3 %	2 %	1 %	0.5 %
C.c.'s of oxygen	5 minutes ...	3.2	4.5	5.7	8.7	13.1	8.5
	5-10 ,, ...	2.0	3.2	3.1	6.0	6.1	1.4
	10-15 ,, ...	1.6	3.1	2.9	5.7	2.4	0.1
	Total .....	6.8	10.8	11.7	20.4	21.6	10.0

Maximum liberation between 1 per cent. and 2 per cent. of  $\text{H}_2\text{O}_2$ .

TABLE Vb

5 c.c.  $\text{H}_2\text{O}_2$  solution + 0.5 c.c. diluted blood (one-quarter strength of that used in V)

		$\text{H}_2\text{O}_2$					
		5 %	4 %	3 %	2 %	1 %	0.5 %
C.c.'s of oxygen	5 minutes ...	0.4	1.1	1.85	2.9	4.2	5.2
	5-10 ,, ...	1.0	1.5	1.35	2.0	4.4	2.8
	10-15 ,, ...	1.1	1.2	1.20	1.9	3.6	2.6
	Total .....	2.5	3.8	4.4	6.8	12.2	10.6

Maximum liberation at 0.5-1 per cent.  $\text{H}_2\text{O}_2$ .

It is evident from a consideration of these results that the optimum concentration of the substrate does vary with the amount of enzyme used.

It might be supposed that in the above experiment the optimum for the strongest blood solution was  $2\frac{1}{2}$  per cent. of  $\text{H}_2\text{O}_2$ , in the second series  $1\frac{1}{4}$  per cent., and in the case of the weakest solution about 0.63 per cent.—i.e., that the optimum concentration of  $\text{H}_2\text{O}_2$  varies directly as the concentration of the enzyme.

The following experiment, however, shows that this is not the case; in this observation a stronger solution of blood was used in the first instance in order to raise the optima somewhat throughout.

TABLE VI

 $\text{H}_2\text{O}_2$  5 c.c. Blood solution 0.5 c.c.

		$\text{H}_2\text{O}_2$								
Dilution of blood solution		5 %	4 %	3 %	2.5 %	2 %	1.75 %	1.5 %	1.25 %	1.0 %
C.c.'s of oxygen in 5 minutes	Strong solution ...	53.5	68.5	61.9	—	41	—	—	—	22.2
	50% dilution of ...	—	30.6	35.0	44.7	41	—	—	—	20.6
	25% ,, ...	—	7.7	10.6	—	20	25.1	24.2	24	19

(It was found that equally comparable results were obtained by measuring the gas evolved during the first five minutes as by measuring that liberated in fifteen minutes.)

The maxima in these three series were :—

With strong enzyme between 3 per cent. and 4 per cent.  $\text{H}_2\text{O}_2$  (mean 3.5).

.. 50 per cent. of enzyme between 2 per cent. and 2.5 per cent.  $\text{H}_2\text{O}_2$  (mean 2.25).

.. 25 per cent. of enzyme between 1.5 and 1.75 per cent.  $\text{H}_2\text{O}_2$  (mean 1.62).

The optimum percentage concentrations of  $\text{H}_2\text{O}_2$  ( $\omega$ ), therefore, do not vary directly as the concentrations of the enzyme ( $\epsilon$ ) but more nearly as their square roots :—

TABLE VIa

$\epsilon$		$\sqrt{\epsilon}$		$\omega$	Ratio	$\frac{\sqrt{\epsilon}}{\omega}$
100	...	10	...	3.5	...	2.86
50	...	7.07	...	2.25	...	3.15
25	...	5	...	1.62	...	3.10

It would, however, be far from wise to express this as a definite law, for the reason that in all probability a different relation would be established when very wide ranges in concentration of catalase were examined. All that can be said is that for the strengths obtained by ordinary dilutions of blood this rule seems to hold approximately.

#### INFLUENCE OF VARYING CONCENTRATIONS OF ENZYMES

If any law connects the activity of solutions of catalase with their concentration, it is certainly somewhat obscure ; no rule seems to be clearly followed at any very wide limits of concentration.

Theoretically, catalase lends itself admirably to the solution of such a problem, since it is easy to observe the times necessary for equal amounts of change, when a constant volume of enzyme solution of different strengths acts on a uniform amount of hydrogen peroxide solution of a fixed percentage concentration: Moreover the end products, oxygen and water, from their nature cannot accumulate and interfere with the reaction, especially when only an early stage of the latter is studied. These conditions would appear to be almost ideal, and the relative activity of any two solutions of catalase would, as indicated by B. Moore (16), be inversely proportional to the times necessary for the evolution of equal volumes of oxygen.

Quite an unexpected factor is introduced, however, by the great difference in the influence of hydrogen peroxide solutions in inducing destruction of catalase when present in varying amounts in the solution; generally speaking the weaker the enzyme the more readily it is destroyed by the substrate. It was eventually found that different laws were followed at different ranges of strength of catalase. These it is believed may be to a large extent explained on taking into account two properties of catalase already mentioned, viz:—

- I. That there are optimum concentrations of hydrogen peroxide which are different for each new strength of enzyme employed.
- II. That when the relative amount of substrate is large, catalase is rapidly changed into an inactive form.

In the experiment given in tabular form below, laked blood was used as the source of the enzyme. The activities of the various admixtures of blood and water were compared by observing the times necessary for the liberation of 10 c.c. of oxygen when the enzyme solution acted on 1 per cent. hydrogen peroxide. After making the necessary calculations it was possible to compare activity with concentration of enzyme.

TABLE VII  
1 per cent.  $H_2O_2$  50 c.c. + 10 c.c. blood solution

Relative concentration of enzyme [ $\epsilon$ ]		Time in seconds to evolve 10 c.c. oxygen [ $t$ ]		Relative activity ( $a$ ) [ $= \frac{2'3}{t} \times 100$ ]
100	...	2'3	...	100
50	...	3'0	...	76'7
40	...	3'6	...	63'8
30	...	4'0	...	57'4
20	...	5'7	...	40'3
15	...	7'0	...	32'7
10	...	10'2	...	22'6
6	...	13'8	...	16'6
4	...	24'2	...	9'5
2	...	65'2	...	3'52
1	...	212'8	...	1'08
0'2	...	1841'0	...	0'125

It would at first seem as though nothing approaching a fixed rule is followed here, and on attempting to analyse the graphic curve relating concentration and activity it was indeed found that no simple mathematical expression could be found to represent the relation.

On closer examination, however, it was found possible to divide the enzyme concentrations into three ranges, for two of which separate formulae were approximately correct.

When we consider the results for enzyme solutions the relative strengths of which range between 100 and 30 in the above table, we see that Schütz's law, which is said to apply for wide limits of concentration in the use of a large number of enzymes, is fairly well adhered to in this case also. The table given below illustrates this:—

TABLE VIIa

Enzyme $\epsilon$		Activity $a$		Ratio $\frac{a}{\sqrt{\epsilon}}$
100	...	100	...	10'0
50	...	76'7	...	10'8
40	...	63'8	...	10'06
30	...	57'4	...	10'50

When, on the other hand, the relative concentrations lay between thirty and four, that is to say, over the widest range of concentrations studied, the activity varied directly as the concentration. This, it may be remarked, is the usual rule followed by inorganic catalysts such as platinum sol.

TABLE VIIb

Enzyme $\epsilon$		Activity $a$		Ratio $\frac{a}{\epsilon}$
30	...	57'4	...	1'91
20	...	40'3	...	2'01
15	...	32'7	...	2'18
10	...	22'6	...	2'26
6	...	16'6	...	2'76
4	...	9'5	...	2'37

Below four the activities rapidly diminish with lessening concentration of enzyme, and no law is followed over any considerable range of concentrations.



These results show what has been already abundantly demonstrated, namely, that considerable caution is to be observed when comparing enzymes with inorganic catalysts (the so-called inorganic ferments). The enzyme molecules from their necessarily great complexity are far more liable to be interfered with by any unusual conditions. It seems not at all unlikely that other enzymes may be found to deviate from the usually expressed laws that relate concentration and activity, if very wide limits were studied. Thus Bayliss (17) has shown that trypsin in dilute solutions exhibits a linear law and not the Schütz law which it is said to follow in more concentrated solutions.

In the case of catalase, (and particularly the catalase of animal origin), this body is so extremely susceptible to disturbing influences that the ranges over which any law holds are necessarily very narrow. We may assume that in the absence of the two interfering factors previously mentioned, the catalysor would follow the law which usually applies to inorganic catalysts, viz., that the activity of the enzyme solution is directly proportional to its concentration.

These interfering factors were at a minimum when the enzyme concentration lay between the numbers thirty and four (the peroxide being in a 1 per cent. solution), and this rule was therefore adhered to over this range.

For stronger solutions of blood, however, the conditions gradually alter, for now the hydrogen peroxide is much below optimum strength, so that the increasing concentrations of enzyme are working at a rapidly increasing disadvantage, as previously shown (Tables VI and VIa). Indeed a point is very soon attained beyond which further increase in the amount of enzyme in solution gives no further activity when measured by the same strength of hydrogen peroxide (1 per cent.). Hence, until this maximum point is reached, though the absolute activity increases with the growing concentration of the enzyme, the relative catalysing power diminishes. The Schütz law is an empirical way of expressing this fact.

Below the relative concentration six in Table VII, another disturbing factor comes into play. The amount of enzyme is now so

small that the substrate is a supra-optimal one ; the catalase accordingly suffers alteration to a rapidly increasing extent with increasing dilution, so that the activity speedily diminishes.

#### THE VELOCITY OF THE REACTION

##### *The Linear Period*

There is abundant evidence that during the early stages in the action of many enzymes there is a period, the duration of which depends upon various conditions, during which the masses of substrate converted in equal intervals of time are approximately equal, i.e., there is a distinct tendency for the conversion of a constant mass of the substance undergoing disintegration, and not of a constant fraction of it. Thus A. J. Brown (18) working with invertase, Armstrong (19) with various sucroclastic enzymes, and Bayliss (20) with trypsin, have found that when the amount of enzyme present is relatively small, the values of the constant  $K = \frac{1}{\theta} \log \frac{a}{a-x}$  steadily increase during the earlier phases of the reaction.

Armstrong (21) has even shown that the sucroclastic action of dilute acids may follow the same course.

Previous investigators have all come to the conclusion that the decomposition of hydrogen peroxide by catalase very closely resembles its breaking down by inorganic catalysts. This is undoubtedly true for the strength of peroxide usually employed, which has generally been diluted to such an extent that the ratio of substrate to enzyme would not be very considerable. Under these conditions the progress of the reaction is such as may be expressed by the usual empirical formula for a monomolecular decomposition.

As might be anticipated the linear period is best shown when the ratio of peroxide to catalase is as large as it can be made without interfering with the action of the latter to any considerable extent ; that it is of great importance that the peroxide be not too concentrated will be evident on recalling the results already submitted (Table III).

The figures in Table VIII are from an experiment in which the 'collection' method was used : the flask containing the reacting substances was surrounded by ice.

TABLE VIII

200 c.c.  $\text{H}_2\text{O}_2$  N/5.68 + 10 c.c. dilute enzyme solution

Time $\theta$	C.c.'s of oxygen (corrected)	$K = \frac{1}{\theta} \log \frac{a}{a-x}$
3	7.95	0.00600
6	12.85	0.00808
9	19.90	0.0111
12	16.30	0.0123
15	12.70	0.0126
18	12.50	0.0130
24	13.60	0.0120

Here we have a progressive rise in the value of  $K$ , which increases for eighteen minutes. The method used has, however, been shown to be open to serious fallacies; these results are given merely to show that the linear period may be rendered evident by this method, which is the one that had been most extensively used until Senter introduced the titration method.

Even after the expiration of the nine or twelve minutes necessary for complete saturation of the solution with oxygen, there is still a distinct tendency for the reaction to remain linear. Thus in the two successive periods ending at the fifteenth and eighteenth minutes practically equal volumes of oxygen are evolved, pointing to the conversion of a nearly constant mass of substrate in each of the two intervals of time.

The next two experiments were performed by the titration method, which is not liable to the errors attendant upon the older method.

TABLE IX

50 c.c. N/50  $\text{H}_2\text{O}_2$  + 50 c.c. extremely dilute enzyme. Temperature  $20^\circ\text{C}$ .

10 c.c. of reaction mixture titrated at stated intervals with approximately N/500  $\text{KMnO}_4$  solution.

$\theta$ (min.)	C/ $\text{H}_2\text{O}_2$	$K$
0	40.90	—
5	16.40	.0794
10	5.70	.0856
15	2.05	.0866
20	0.90	.0829
30	0.55	.0624

TABLE X

100 c.c. N/100  $\text{H}_2\text{O}_2$  + 100 c.c. of enzyme solution (strength one-half of that used in previous experiments). Temperature  $20^\circ \text{C}$ .

$\theta$ (min.)	C/ $\text{H}_2\text{O}_2$	K
0	55.35	—
5	45.25	.0175
10	35.80	.0189
20	23.50	.0186
30	15.85	.0181
40	11.10	.0174

In both experiments an increase in the values of K is noticeable early in the reaction. The rise is much more marked in the observation in which both solutions were more concentrated, in fact the second series of figures might be said to show a very fair agreement with the requirements for a monomolecular reaction.

Only one more result need be quoted.

In the following experiment the ratio of substrate was very great, and when the observation was carried out at a temperature of  $20^\circ \text{C}$ . the values of K rapidly fell off from the very commencement, but at  $10^\circ \text{C}$ . the figures below were obtained.

TABLE XI

200 c.c. N/10  $\text{H}_2\text{O}_2$  + 1 c.c. dilute enzyme. Temperature  $10^\circ \text{C}$ .

$\theta$ min.	C/ $\text{H}_2\text{O}_2$	K
0	15.77	—
1	14.53	0.0356
2	12.92	0.0428
5	11.60	0.0267
10	9.35	0.0227
15	7.70	0.0207
20	6.45	0.0194
30	4.70	0.0175

The increase in the value of the constant very rapidly ceased, even under fairly favourable conditions. Parallel experiments gave the same results, so that there can be no doubt that the constant did actually rise.



When the amount of peroxide was still further increased, and especially if the temperature was not very low, the velocity rapidly fell off right from the commencement of the action. Tables XII and XIII illustrate this.

TABLE XII

25 c.c. N/10  $\text{H}_2\text{O}_2$  + 5 c.c. extremely dilute enzyme. Temperature  $14^\circ \text{C}$ .

$\theta$ min.	C/ $\text{H}_2\text{O}_2$	K
0	34.1	—
0.25	33.1	0.0517
0.5	32.9	0.0311
0.75	32.6	0.0260
1.0	32.25	0.0242
1.5	31.5	0.0230

TABLE XIII

250 c.c. N/10  $\text{H}_2\text{O}_2$  + 5 c.c. extremely dilute enzyme. Temperature  $20^\circ \text{C}$ .

$\theta$ min.	C/ $\text{H}_2\text{O}_2$	K
0	25.70	—
1	24.80	0.0155
3	23.80	0.0111
5	22.65	0.0109
10	21.70	0.0073
15	21.00	0.0058
20	20.45	0.0050
30	19.75	0.0038
40	18.70	0.0034

It was thought that further demonstration of the linear period could be furnished by comparing the times necessary for equal initial amounts of catalase to produce equal small amounts of change in solutions of hydrogen peroxide of varying concentration. If there is a tendency for the conversion of a constant mass the times should tend to be equal over a fairly wide range of concentration of peroxide. On the other hand, supposing that a constant fraction is converted in unit time, then the times would vary very much, e.g., a certain amount of change wrought in a 3 per cent. solution in say four minutes, would require about 9.1 minutes for its completion when a  $1\frac{1}{2}$  per cent. solution of hydrogen peroxide was employed.

The following experiment is an example of the results obtained.

TABLE XIV

10 c.c.  $\text{H}_2\text{O}_2$  + 1 c.c. dilute laked blood. Temperature  $13^\circ \text{C}$ .

	$\text{H}_2\text{O}_2$ %								
Percentage of $\text{H}_2\text{O}_2$	10	5	3	$2\frac{1}{2}$	2	$1\frac{1}{2}$	1	$\frac{3}{4}$	$\frac{1}{2}$
Time in min. to evolve 10 c.c. oxygen	8	6'10	2'20	2'30	2'31	2'50	2'55	3'20	4'30

The optimum concentration is apparently at about 3 per cent. Between 3 per cent. and 1 per cent. the times are very nearly uniform, much more so than could have been the case had the usual law of mass action been closely followed.

These results can leave no doubt as to the existence of a linear period early in the course of the decomposition. The initial velocity is, however, very soon diminished, especially when strong solutions of hydrogen peroxide are employed. There are strong grounds for believing that the effect of a high proportion of substrate is to induce a change in the catalase molecule, whereby the latter is rendered unfit for carrying on further catalysis. This transformation of the enzyme into an inert form is generally said to be due to a combination of the enzyme with the oxygen set free by the decomposition; if so, then the union must occur either when the oxygen is yet in a nascent condition, or subsequently as a result of the accumulation of the free gas.

The latter alternative may be dismissed, seeing that when supra-optimal concentrations of the substrate are used very little oxygen is evolved at any stage of the reaction.

Nor does the former hypothesis seem more pregnant since according to Schaffer (22) the oxygen is liberated in a molecular condition and is incapable of bringing about oxidations. (This very important point, however, needs re-investigation).

It would therefore seem as though the only probability is that the peroxide itself exerted some chemical action upon the enzyme.

Speculations as to the nature of this influence are perhaps rather premature, but two possibilities suggest themselves.

In the first place, a solution of hydrogen peroxide, even when quite pure, exhibits many of the properties of an acid. It has been

abundantly proved by Senter (23) that solutions of acids and of substances possessing oxidising properties cause a depression of the catalysis by haemase quite apart from any oxidising effect which these substances may produce. When we consider that hydrogen peroxide is a substance which belongs to both classes, we might reasonably suppose that when employed in large excess it might interfere with the steady progress of the reaction. It is also a significant fact that alkalis greatly accelerate the velocity of the change. The other possibility is that the catalase is oxidised by the nascent oxygen set free by the spontaneous decomposition of the hydrogen peroxide. This undoubtedly occurs normally in any unacidified solution of the substance, and may be potent in bringing about destruction of the specific properties of catalase.

#### THE LOGARITHMIC PERIOD

When a due proportionality is established between substrate and enzyme, whether this be after the persistence of the linear period for some time, or whether the necessary conditions exist at the moment of mixing the two solutions, the law of mass action is followed. The values of  $K$  are now constant. The logarithmic phase is well seen when the substrate used is in very dilute solution and the amount of enzyme not very small.

In the following experiment (Table XV) the values of  $K$  are practically constant for fifteen minutes, and it would seem as though the reaction was indeed a true monomolecular one.

TABLE XV

400 c.c. N/100  $H_2O_2$  + 200 c.c. dilute enzyme (ice thermostat)

$\theta$ (min.)	C/ $H_2O_2$	$K$
0	10.35	—
1	9.32	0.0455
2	8.45	0.0440
3	7.65	0.0438
4	6.90	0.0440
5	6.20	0.0445
10	4.05	0.0407
15	2.55	0.0406
		Mean = 0.0433

## THE PERIOD OF MARKED SLACKENING

As a result of the progressive destruction of the catalase, the logarithmic course of the reaction becomes modified and depressed so that the values of  $K$  fall. If the slowing factor is very powerful the first two portions, the linear and logarithmic, are omitted, and the velocity rapidly falls from the very commencement (*vide* Tables XII and XIII). Under any circumstances this phase will become conspicuous sooner or later, and that it is due to, or at least accompanies, extensive destruction of catalase may be shown according to the method of v. Laer (*loc. cit.*). After the lapse of twenty-four minutes in the experiment given in Table VIII the original concentration of the substrate was restored by the addition of an amount of 30 per cent.  $H_2O_2$ , which was equivalent to the oxygen given off. The volumes of oxygen evolved under the new conditions were measured and compared with those given off after the same intervals of time in the first series of readings. The result is given below.

TABLE XVI

Time	Reading in 1st series	Reading in 2nd series	Ratio = residual catalase
9 min.	40.7	23.2	0.57
12 „	57.0	30.3	0.53
18 „	82.2	39.3	0.47

The amount of catalase has been reduced to one-half by the end of twenty-four minutes only, showing that animal catalase is very readily destroyed.

Van Laer has, by the application of a method of graphic analysis, eliminated this variability from the results and substituted a number which represents an average amount of catalase throughout the reaction. By this method he has shown that the net result of an action proceeding for 140 minutes is in accordance with the requirements of the law of mass action. This result, however, gives the net result merely, and cannot tell us anything about the fluctuations which the velocity suffers from time to time. Indeed v. Laer's own experiments certainly show the three periods in the reaction admirably, since the yeast catalase is well adapted to long observations.



## CONCLUSIONS

The results of the author's experiments on the reaction velocity of hydrogen peroxide catalysis may, it is believed, be explained by the hypothesis first suggested by Professor Adrian Brown, and subsequently by Armstrong, and now generally known as the 'active system' theory. In the presence of a large excess of substrate the magnitude of the active system would be constant and the reaction rectilinear. At a later stage the formation of active systems would be a function of the total mass of substrate, and hence the curve representing the reaction would be logarithmic.

There is little doubt, however, that an apparently logarithmic course is developed earlier than should be the case very frequently on account of the destruction of a certain amount of the catalase during the first stages of the reaction; for this same reason also the logarithmic period is by no means well sustained, since with a diminishing amount of enzyme the magnitude of the active system will, as Armstrong has shown, be a function of both substrate and enzyme. For this reason the value of  $K$  will fall wherever there is extensive removal of the ferment. Whether there is an equilibrium point in the reaction, and whether the action is or is not reversible, it is difficult at present to determine. There certainly seems to be a balanced point, but this is very near to zero, and the existence of any reversibility factor is very doubtful owing to the gaseous nature of the principal end product. Comparative experiments carried out in atmospheres of oxygen under varying pressures certainly indicate a slight slowing of the reaction when the oxygen tension in the apparatus is considerably increased, but nothing very definite has yet been proved in this direction, chiefly on account of experimental difficulties.

## SUMMARY OF RESULTS

1. The two methods in general use are compared, and the fallacies of one of them demonstrated.
2. For each concentration of enzyme there is an 'optimum'

concentration of hydrogen peroxide. The optima do not vary directly as the enzyme concentrations, but more nearly as their square roots.

3. No single law expresses the relation between the concentration and activity of the enzyme solutions. Above a certain point increase in the strength of the catalase solution is without effect. For some distance from this point the Schütz law is followed. With increasing dilutions of ferment there is a linear relation between the strength of the enzyme solution and the amount of catalysis it can induce. With very great dilutions there is a rapid and irregular departure from this rule. These results are briefly discussed.

4. The velocity with which the catalysis occurs is studied; the three periods common to many enzymatic actions are found to be present. These periods are (*a*) the Rectilinear, (*b*) the Logarithmic, (*c*) the infra-Logarithmic period.

5. These results are explained with the aid of the usual hypothesis of an 'active system,' and the possibility of the action being reversible are very briefly indicated.

In conclusion, I would tender my very hearty thanks to Professor Carlier for his kindly support and direction during the progress of this research, and to Professor Adrian J. Brown for the many valuable suggestions which he was good enough to offer.

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## ON THE INFLUENCE OF KREATININ IN MODIFYING CERTAIN REACTIONS OF SUGAR IN URINE

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In a former article<sup>1</sup> it was pointed out that the principal normal constituent of urine which markedly interferes with the results obtained on applying Fehling's test for the detection of small amounts of sugar is kreatinin. The constant presence of kreatinin in urine completely invalidates the test when used in the ordinary way for the detection of minute amounts of sugar, and it is probably for this reason that the constant physiological occurrence of sugar in urine has for so long been doubted or denied. Kreatin acts in the same way only it is much less powerful. Since, according to Folin,<sup>2</sup> kreatin may appear in the urine in large amounts, especially after a meat diet, it is likely that in certain urines kreatin may largely enhance the effects of kreatinin; in the majority of urines, however, the amount of kreatin appears to be small and its direct effect of minor importance.

Kreatinin is capable of acting in different ways when heated with Fehling's solution to which has been added small quantities of some reducing agent such as sugar. It is itself capable of slowly reducing Fehling's solution and at the same time possesses the property of holding the reduced suboxide in solution, thus apparently inhibiting its own reducing action as well as that of any other reducing constituent that may be present.

When, as often happens in urine, the amount of kreatinin present is barely sufficient to hold in solution the reduced suboxide obtained by the action of small amounts of sugar as well as by that of the kreatinin itself, the result is so modified as to render its interpretation doubtful. Those ambiguous reactions in which a more or less opalescent solution tending immediately or after cooling to show an indistinct greenish-

1. *Bio-Chemical Journal*, Vol. I, p. 111.

2. *Zeit. f. Physiol. Chem.*, Bd. XLI., S. 223 (1904).



yellow precipitate suspended in the liquid, have hitherto constituted one of the chief objections to this otherwise excellent test; if, however, the above explanation of this phenomenon be kept in view it may help in deciding what value is to be attached to this characteristic reaction in any individual case. In the absence of drug treatment these opalescent solutions generally indicate the presence of sugar in amount somewhat beyond the normal percentage; it is obvious that the amount of urine actually passed per diem must be taken into account in estimating the significance of such reaction. On the other hand, the absence of kreatinin or some retarding factor in urine would also interfere with Fehling's test; for the normal amount of uric acid alone, exclusive of other reducing agents present, gives a fairly marked Fehling's reaction.

#### ON THE MANNER IN WHICH KREATININ INHIBITS THE USUAL EFFECT OF SMALL QUANTITIES OF SUGAR ON FEHLING'S SOLUTION

While it seems certain that the only important nitrogenous constituent of normal urine that exerts a marked influence in retarding the effects of Fehling's solution is kreatinin, there are obviously two ways in which this might be accomplished: (1) the alkaline part of Fehling's solution might act on the kreatinin and generate ammonia, which, if evolved in sufficient quantity, would hold the reduced suboxide in solution; or (2) the kreatinin, as such, possesses the property of holding the suboxide in solution.

That the first hypothesis is incorrect is evident from the following considerations and experiments.

(a) About 2.5 mgr. kreatinin is required to prevent precipitation of 1 c.c. of .1 per cent. aqueous dextrose solution when mixed with 1 c.c. Fehling's solution. If we assume for purposes of experiment that all the nitrogen of the kreatinin goes to form ammonia, the total amount of  $\text{NH}_3$  resulting from the decomposition of this amount of kreatinin is only about 1.12 mgr.

$$\underset{\text{(M.W. 113)}}{\text{C}_4\text{H}_7\text{N}_3\text{O}} = 3(\text{NH}_3) = \frac{51}{113} \times \frac{5}{2} = 1.12 \text{ mgr.}$$

A reference to the following tables shows that a much greater amount of ammonia than 1.12 mgr. is necessary to prevent the appearance of a precipitate with a solution of .1 per cent. dextrose.

TABLE I (AMMONIA)

	Ammonium hydrate pur. (.880) (1 in 10)	Ammonia calculated as nitrogen in mgrs.	Results
1 c.c. .1 % dextrose solution + 1 c.c. Fehling's solution boiled with	1 drop	1.5 mgr.	Immediate precipitate
	2 drops	3.0 "	" "
	4 "	6.0 "	" "
	6 "	9.0 "	pp. after few seconds boiling
	8 "	12.0 "	" " "
	10 "	15.0 "	pp. after 6-10 seconds boiling
	12 "	18.0 "	pp. after 8-12 " "
	16 "	24.0 "	pp. after little time

In these cases the cause of the precipitate being produced after boiling for a few seconds was not due to the fact of the ammonia being driven from the hot liquid, for ammonia still continued to come off in great abundance long after a distinct precipitate had formed. It will be noticed that such a large amount as 24 mgrs. of ammonia is insufficient to prevent a reaction for more than a few seconds with .1 per cent. dextrose solution, whereas 2.5 mgrs. kreatinin, with a total possible evolution corresponding to 1.12 mgr.  $\text{NH}_3$ , entirely prevents precipitation even after fairly prolonged boiling.

TABLE II (KREATININ)

	Kreatinin	Total N calculated as ammonia	Results
1 c.c. .1 % dextrose solution + 1 c.c. Fehling's solution boiled with	1 mgr.	0.45 mgr.	Opalescent solution
	2 mgrs.	0.90 "	Slight reaction after half minute
	2½ "	1.12 mgrs.	No pp. after long boiling (one minute)
	3 "	1.35 "	" " "
	4 "	1.80 "	" " " sol. slightly greenish-blue

(b) Several mixtures of kreatinin and alkaline part of Fehling's solution were distilled for periods varying from three to eight minutes and the ammonia calculated in the ordinary way by  $\text{N}/_{10}\text{NaOH}$ ;

after several minutes boiling the amount of ammonia did not nearly correspond to the total amount of nitrogen present in the kreatinin, the quantity evolved being absolutely insufficient to exert any inhibiting influence on even a minute quantity of sugar; a certain amount of ammonia was always obtained, but it was evolved very slowly and in small quantity.

(c) An examination of the liquid used in the above experiments showed that it still possessed a fairly marked inhibitory effect after five minutes boiling; this proved that the kreatinin had not been all destroyed in this time.

(d) Distillation for several minutes of small quantities of normal urine mixed with alkali, and giving a strong kreatinin reaction, resulted in the production of ammonia in quantity insufficient to exert any marked retarding effect.

From these observations it seems obvious that the influence of the alkali of Fehling's solution on the nitrogenous constituents of the urine generating free ammonia is quite insufficient to explain the retarding effects described above; any ammonia generated undoubtedly helps, but only to a limited extent, for from its slow evolution there cannot be more than a comparatively small quantity present in the boiling liquid at any particular moment. Urea in the percentage in which it occurs in urine yields much more ammonia than the kreatinin of an equal amount of urine, and yet it possesses no apparent retarding effects; for a very dilute solution of dextrose (less than .01 per cent.) introduced into a 2.3 per cent. urea solution and mixed with equal parts of Fehling's solution gives quite a distinct reaction. Kreatinin therefore acts by holding the reduced suboxide in solution.

#### ON THE MODIFICATION OF COLOUR OBTAINED BY FEHLING'S TEST IN URINE CONTAINING SMALL AMOUNTS OF DEXTROSE

If a weak solution of dextrose (say .4 per cent.) in distilled water is boiled with an equal amount of Fehling's solution a *red* precipitate of cuprous oxide is quickly deposited; if the same amount of sugar be added to normal urine and Fehling's solution used as before the resulting precipitate is distinctly *yellow*, and differs markedly in appearance

from that obtained from dextrose in distilled water; if a little kreatinin be added to watery sugar solution a yellow precipitate is obtained, exactly like that obtained from the urinary solution. This modification in colour is generally admitted to be caused by kreatinin, and is generally explained in the text books as being due to a deposition of cuprous hydrate ( $\text{Cu}_2\text{OH}_2\text{O}$ ). A quotation taken at random from a popular clinical text book is as follows: 'On boiling, the blue cupric hydrate is reduced to cuprous hydrate, which is not capable of being held in solution and accordingly appears as a yellow precipitate, which may be further dehydrated to cuprous oxide ( $\text{Cu}_2\text{O}$ ) which is red.' This explanation seems to be generally accepted, it being assumed that a yellow precipitate indicates the lower *hydrate*, whereas a distinctly red one indicates the lower *oxide*. Thus, almost every urine containing enough sugar to give a precipitate with Fehling's test, apparently gives the yellow hydrate. The lower hydrate of copper is, however, one of the most unstable of chemical compounds, and it appears strange that it should appear so frequently in urine testing with Fehling's solution. This yellow substance is by no means unstable, for it often remains yellow for hours or even days, as can be easily observed if some of it be poured into a quantity of distilled water and put aside; the ordinary cuprous hydrate under similar conditions very soon changes, and this at once suggests a doubt in regard to the constitution of the yellow precipitate obtained from urine.

It would appear that both the red and yellow precipitate are really the same chemical substance—the lower oxide of copper ( $\text{Cu}_2\text{O}$ ).<sup>1</sup> The difference in colour does not necessarily indicate a change in the chemical composition, but seems to be dependent on the state of subdivision of the precipitate; if the precipitate is finely subdivided it has a yellow colour; if comparatively coarse it is red. This can be demonstrated by examining different precipitates under the microscope. The red precipitate is seen to be much more coarsely granular,

1. As far as I am aware the first to suggest that the yellow precipitate obtained on heating certain saccharine urines with Fehling's solution is probably not the cuprous hydrate, but really the modified cuprous oxide, was Dr Fraser of the Aberdeen Royal Infirmary, who informs me that he has taught this for many years.



being quite different in this respect from the yellow. If the red oxide be taken and thoroughly dried and then well ground in a mortar, it gradually assumes a yellow appearance; on subsequent mixture with water a yellowish fluid is obtained. The red precipitate can be comparatively easily separated from the urine by filtration, but often it is very difficult to separate a markedly yellow one owing to the fineness of the particles. If the precipitate is very finely divided it appears as a greenish opalescence which may take many hours to fall down, and even by centrifugalising it is difficult to separate it from the liquid in which it is suspended; a distinctly yellow precipitate, on the other hand, can be fairly easily thrown down by the centrifuge, and the red one separates out very readily indeed. If a few drops of urine giving, when tested in the ordinary way, a yellow precipitate, be dropped into a fairly large quantity of boiling Fehling's solution, a red precipitate separates out—in other words, a urine giving a yellow precipitate when heated with equal parts of Fehling's solution gives a red precipitate when heated with excess.

All the above modifications are explained by the fact that the state of subdivision of the precipitate of cuprous oxide obtained when a weak solution of dextrose, or urine containing a small percentage of sugar, is heated with Fehling's solution depends on the relative amount of kreatinin present. When the amount of the latter is just insufficient to inhibit the influence of the sugar present, a very finely divided precipitate of cuprous oxide separates out, which may show merely as an opalescent milky solution; the addition of just a trace of sugar to the solution makes it still more difficult for the kreatinin to hold the suboxide in solution, and it is deposited in a slightly coarser form than before, as indicated by an opalescent solution in which the eye can detect a faint precipitate on careful observation; at this stage the general colour of the solution is a dirty greenish yellow; the addition of extra traces of sugar makes the precipitate coarser and coarser, and soon a markedly yellow liquid showing a distinct precipitate is obtained. If still more sugar be added a red precipitate appears, which soon settles to the bottom of the tube, and is seen by microscopic examination to be fairly granular.

The following table indicates the changes observed in the precipitate obtained from a urine, to which different strengths of sugar has been added.

Amount of sugar added per cent.	Time required for boiling	Appearance	Time required to separate precipitate by centrifuge <sup>1</sup>	Microscopical appearance	Filtration
Trace	2½ minutes	Greenish opalescence	2 hours	Very many minute particles with few large ones	Impossible
·015 %	1¼ „	Greenish pp	1 „	„	Extremely difficult
·04 %	1¼ „	Greenish-yellow pp.	½ hour	Many fine : fair number coarse	Very difficult
·1 %	30 seconds	„	10 minutes	„	Fairly difficult
·4 %	10 „	Yellow pp.	10 „	Mixture of very fine and fairly coarse	Rather difficult to get quite clear solution
3·0 %	Immediately	Red pp.	2½ „	Large, coarse, with comparatively few fine granules	Easy to get clear filtrate

By carefully adjusting the relative amounts of kreatinin and sugar present in a liquid all shades of colour from greenish-yellow to bright red can be obtained after boiling with Fehling's solution. A small quantity of sugar (say ·15 per cent.) added to distilled water gives a more or less dirty yellow appearance, but this is caused by the red particles floating in the blue liquid, and here there is really no fine subdivision, for on centrifugalising the liquid a bright red precipitate is obtained, and the blue liquid appears quite free from suspended matter; the centrifuge completely separates the precipitate in a very short time, and in fact, if left to stand, it generally falls in from five to ten minutes. A trace of kreatinin added previous to boiling results in the precipitate being quite yellow as described above.

Thus it would seem that here we have an explanation of all the

1. The centrifuge used was purposely not a rapid one.

different coloured precipitates obtained on boiling urines containing small amounts of sugar with Fehling's solution. In urine the first part of the suboxide that separates out is exceedingly fine; as the kreatinin loses its effect the precipitate becomes more coarsely granular, till ultimately the ordinary red is obtained. In urine we have the results of all these stages depending on the relative amounts of sugar and kreatinin present, and thus the colour shows notable differences.

In order to demonstrate the different stages it is only necessary to add a trace of sugar to urine and boil till the first sign of opalescence is detected. Then centrifugalise for some hours and add a second trace of sugar to the blue liquid obtained; heat, and again centrifugalise. This time the precipitate obtained is slightly more yellow than at first, and if this be repeated for a few times the precipitate will ultimately appear quite red. Exactly the same phenomena can be seen with kreatinin itself. Kreatinin tends slowly to reduce Fehling's solution and after some time an opalescent, slightly greenish, milky liquid is obtained; filtration results in the filter paper being stained green, the filtrate, however, being still opalescent. If boiling is continued for some time longer and the liquid again filtered, the stain is now seen to be distinctly yellow; and a third filtration, after somewhat prolonged boiling, results in the filter paper being distinctly red. The following are notes of an experiment with kreatinin.

TABLE IV

Weak solution of kreatinin with equal parts of Fehling's solution	{	Boiled for 5 minutes = greenish opalescent fluid; after filtering still opalescent, but filter paper stained greenish.
		„ 6 minutes = Filter paper stained greenish-yellow; liquid still opalescent.
		„ 10 minutes = Filter paper stained distinctly red, showing granular pp. of $\text{Cu}_2\text{O}$ . Filtrate still opalescent.

THE SODIUM-NITRO-PRUSSIDE TEST AND THE PICRIC ACID  
TEST FOR KREATININ

In performing experiments to determine the reduction of kreatinin after boiling in alkaline solution, it was necessary to make use of some fairly delicate indicator. Picric acid in alkaline solution when used in the cold, while very definite in regard to strong kreatinin solutions, was found to be worthless for small amounts as no reaction was obtained; on the other hand, it was equally unsatisfactory after heating, for a mixture of picric acid and alkali, in the absence of all other substances, gives on heating a more or less distinct red colour. Nitro-prusside of sodium in alkaline solution was found, however, to be a very delicate test, and with care such a small quantity as  $\cdot 012$  per cent. kreatinin in solution could be detected with certainty. It is generally stated that the test is not applicable to a solution containing less than  $\cdot 03$  per cent. kreatinin, but by the use of strong solution of sodium-nitro-prusside there was no difficulty in detecting even less than  $\cdot 012$  per cent., especially when a control test with a solution of alkali and nitro-prusside without kreatinin was made use of.

This reaction of nitro-prusside of sodium with kreatinin in alkaline solution was pointed out by Weyl<sup>1</sup> many years ago. Salkowski<sup>2</sup> showed later on that the addition of acetic acid to the red coloured liquid obtained by Weyl's reaction, and boiling, it turns greenish or greenish-blue, and on standing, Prussian blue separates. The application of this reaction as a test for acetone has been utilised by Legal<sup>3</sup> and Le Nobel,<sup>4</sup> and a considerable amount of confusion seems to prevail in regard to the exact reactions given by kreatinin and acetone respectively. That the addition of a few drops of sodium nitro-prusside and sodium hydrate to a solution containing either acetone or kreatinin will in each case produce a ruby-red colour, which changes to yellow on standing or heating, is generally acknowledged, but the

1. *Berichte, der Deutsch. Chem. Gesellsch.*, 1878, p. 228.

2. Salkowski, *Zeitschr. f. Physiol. Chem.*, 1880.

3. *Pharm. Journal* [5], XVIII, 206.

4. *Chem. Centralb.*, 1884, p. 626.



subsequent action of acidulation and heat has given rise to many apparently contradictory statements.

In regard to acetone, Legal<sup>1</sup> states that a little acetic acid added to the yellow liquid produces a deep violet colouration.

According to Le Nobel,<sup>2</sup> a greenish-blue or violet colour is developed on boiling after the addition of an acid.

Allen<sup>3</sup> states that 'acetone gives a ruby-red colour with Weyl's test, though no blue colour can be obtained on acidulation, acetic acid merely restoring the yellow to red.'

Milroy<sup>4</sup> states that acetone and kreatinin give a somewhat similar reaction with Weyl's test.

Dixon Mann<sup>5</sup> says 'acetone gives a red colour with Weyl's test but not the subsequent blue.'

In another text book<sup>6</sup> the following statement is made, 'acetone gives a red colour with the nitro-prusside and alkali which, however, does not become yellow on standing or even after adding acetic acid.'

In performing Weyl's test for kreatinin it is well known that the red colour obtained changes very quickly to yellow on the addition of heat: in applying the test it is very important that the liquid be not subjected to heat *before* adding acetic acid as is sometimes done, for the nitro-prusside of sodium itself, when heated in the presence of an alkali, undergoes a change and yields a very marked greenish or greenish-blue solution on the addition of a small quantity of acetic or other acid. The marked greenish-blue colour so easily obtained on the addition of acetic acid to urine which has been heated with sodium nitro-prusside and an alkali, depends largely on the interaction between these reagents, and is not simply due to the influence of kreatinin. This action of sodium nitro-prusside and alkali seems to afford an explanation of the contradictory nature of many statements made in regard to the acidulation and heat part of this test.

1. Loc. cit.

2. Loc. cit.

3. 'Chemistry of Urine,' 1895, p. 158.

4. 'Practical Physiological Chemistry,' 1904, p. 106.

5. 'Physiology and Pathology of the Urine,' 1904, p. 47.

6. 'Practical Physiology' (Beddard, Edkins, Hill, Macleod, Pembrey) 1902, p. 261.

That heat is sometimes applied before the addition of the acid is amply proved by the statements of some investigators.

Tests performed with a chemically pure sample of acetone showed that acetone and kreatinin gave practically the same result so far as the nitro-prusside and alkali reaction is concerned—a ruby-red colour gradually changing to yellow. With a comparatively dilute solution of acetone the addition of a few drops of strong acetic acid gives, on agitation of the liquid, a distinct purplish colour; this colour is destroyed by heat, and a clear or slightly greenish-blue liquid is left, which gradually becomes of a deeper tint. The addition of excess of acid tends to destroy this purple or violet colour; subsequent heating, however, still gives a greenish colouration.

With a stronger solution of acetone (say .5 per cent.) the addition of a comparatively large amount of acetic acid does not destroy the violet colour produced; on heating, however, it is very quickly destroyed, and a very marked greenish-blue colour takes its place. Thus it is obvious that both kreatinin and acetone give practically the same result in regard to the formation of a greenish-blue or blue liquid.

The only difference in the test is that a fair amount of acetic acid added to the yellow liquid obtained in the second stage of the nitro-prusside reaction, gives, in the case of kreatinin, a *clear amber colour* (provided care is taken that the mixture be not previously heated), whereas with acetone a *purple* or *violet* colour is produced which, in weak solutions, tends to be destroyed by excess of the acid.

Weyl's reaction is, of course, positive in every urine on account of the kreatinin present, but a little care in the application of the test renders the detection of acetone comparatively easy. The red coloured liquid obtained after alkaline nitro-prusside should be slightly acidified with acetic acid and shaken, and if a clear light-amber coloured liquid is now obtained the red colour is due to kreatinin; if the acidified liquid is slightly red or purple acetone is present as well. Heating in each case gives the same result—a green or greenish-blue liquid.

THE ACTION OF THE ALKALINE PART OF FEHLING'S SOLUTION  
ON KREATININ

To determine the changes which kreatinin undergoes when boiled with Fehling's solution, a definite amount of kreatinin was boiled for varying periods in a 4 per cent. sodium hydrate (watery) solution. Samples of the boiled liquid were obtained, and of these 1 c.c. was taken and four drops of a 15 per cent. solution of nitro-prusside of sodium added, and the mixture shaken. Numerous tests were made in order to ascertain how many drops of the original kreatinin solution (made up to 1 c.c. with 4 per cent. sodium hydrate solution) gave the same colour, and faded at the same time as the boiled solution.

As an example, it was found that two drops of a 1 per cent. solution of kreatinin added to fourteen drops of 4 per cent. sodium hydrate solution (sixteen drops from the pipettes used exactly corresponded to 1 c.c.) gave with nitro-prusside of sodium a red colour which faded in about twelve seconds. This 1 per cent. solution was boiled for about eighteen minutes, and it was then found that 1 c.c. of it gave a red colour similar to the original solution, this red colour fading in about twelve to fifteen seconds. From this it was obvious that 1 c.c. of the boiled kreatinin solution contained the same amount of kreatinin as two drops of the original solution. Two drops of the original solution contained 125 mgr. kreatinin; therefore, by boiling for eighteen minutes the kreatinin solution was reduced from a strength of 1 mgr. per c.c.—a reduction of about 85 per cent. The following notes of one of the experiments show the general change.

TABLE V

Kreatinin sol. 50 mgr. in 50 c.c. water + 2 grms. sodium hydrate

Time boiled		Amount (in drops) of original solution made up to 1 c.c. with 40 per cent. sodium hydrate giving reaction similar to 1 c.c. boiled kreatinin solution		No. of mgrs. present in 1 c.c. of kreatinin solution after boiling (original solution = 1 mgr. per c.c.)
3 minutes	...	9-10 drops	...	0'60 mgr. per c.c.
6 "	...	6-7 "	...	0'40 " "
10 "	...	3-4 "	...	0'23 " "
12 "	...	3 "	...	0'20 " "
17 "	...	2-3 "	...	0'16 " "
25 "	...	1-2 "	...	0'1 " "
30 "	...	Trace	...	—

Similar experiments bear out the above results; viz. :—that about 50 per cent. of a weak kreatinin solution is changed after boiling from four to seven minutes in the presence of caustic alkali in half the strength present in Fehling's solution (4 per cent.). In from ten to fifteen minutes about 80 per cent. of the kreatinin undergoes change, and after thirty minutes only a trace is present.

Another method was adopted in which the inhibitory power of a known amount of kreatinin on a weak sugar solution was determined. The kreatinin solution was then boiled with alkali for some time, and its inhibitory power again determined; the general results agreed with those obtained by means of the nitro-prusside reaction.

That some of the kreatinin remains as kreatin is likely from the fact that a solution boiled for fifteen minutes or so has a somewhat greater inhibitory effect than can be accounted for by the unchanged kreatinin present. This inhibitory power is, however, not nearly equivalent to what might be expected from a solution containing all the changed kreatinin in the form of kreatin, and the only available explanation is that a fair amount of the kreatinin has been destroyed while a certain amount is still represented by kreatin. Kreatin, when boiled with caustic alkali, is not nearly so quickly changed as kreatinin, and the first step here is probably the formation of sarkosin. Sarkosin possesses no apparent inhibitory effect on the reducing action of copper solutions by sugar, but it possesses fairly distinct reducing properties, and acts in a very short time. Thus, a urine boiled for some time with Fehling's solution contains mixtures of kreatinin, kreatin and sarkosin, and the longer it is boiled (within certain limits) the greater tendency there is for an increase in the latter ingredients and a decrease in kreatinin. Since kreatin is not very quickly acted on in alkaline solution, it is obvious that in boiling urine with Fehling's solution any kreatin derived from kreatinin will not tend to change very rapidly.

An attempt was made to determine the action of caustic alkali on urine by adding some sodium hydrate to urine, and after boiling for some time, testing the urine for kreatinin with sodium-nitro-prusside as described above. This was found to be quite impracticable,



for the action of boiling alkali and urine generates some substance (probably an acetone-like body derived from the carbo-hydrate material normally present) which, with sodium-nitro-prusside, gives the same reaction as kreatinin, and quite obscures the test for the detection of small amounts of this substance.

Another interesting point observed on boiling urine with caustic alkali may be mentioned here. After boiling for a very short time with alkali of moderate strength, the ordinary carbo-hydrate matter of urine seems to be completely destroyed. Urine having a reducing action equivalent to a solution containing .1 per cent. dextrose, was, after boiling for twenty seconds with 3 per cent. sodium hydrate, found to contain not a trace of carbohydrate substance as indicated by the most delicate tests for carbohydrate material. A .1 per cent. solution of dextrose in water containing from 2 to 3 per cent. sodium hydrate was found to be absolutely free from dextrose after boiling for from ten to fifteen seconds. In this way a urine can be rapidly freed from carbohydrate matter. While it is a well known fact that boiling or hot alkali destroys sugar, it would seem that the extreme facility with which this is accomplished is hardly appreciated.

#### THE ACTION OF THE ALKALINE PART OF FEHLING'S SOLUTION ON KREATIN

Kreatin, like kreatinin, reduces Fehling's solution only after prolonged boiling, and since some of the kreatinin of urine is changed into kreatin when boiled with Fehling's solution, some experiments were made to determine the effect on kreatin of boiling with an alkali. The inhibitory effect of the original kreatin solution was determined and compared with a sample which had been subjected to boiling with sodium hydrate for a definite time. In experiments a weak solution of sugar was used, and 1 c.c. of this mixed with 1 c.c. Fehling's solution; on heating a distinct reaction was obtained, which could be postponed and modified by the addition (before boiling) of a few drops of kreatin solution. In one such experiment it was found that 1 c.c. sugar solution + 1 c.c. Fehling's solution + three drops kreatin

solution, on boiling for ten seconds, gave, on standing for forty seconds, a distinct greenish opalescence. After boiling this kreatin solution (containing 4 per cent. sodium hydrate), tests were made in order to ascertain how many drops of the boiled kreatin solution gave a result as nearly as possible corresponding to the effect of three drops of the original solution. The following results were obtained.

Time original solution of kreatin boiled with alkali			No. of drops equivalent to three of original solution		Remarks
3 minutes	...		3	...	No appreciable change caused by boiling
6	„	...	4	...	Reaction with four drops some- what less distinct than with three original solution.
15	„	...	6	...	—
22	„	...	8	...	—

From the above it will be seen that kreatin is changed very much more slowly than kreatinin, when boiled with an alkali. After boiling for twenty-two to twenty-five minutes the solution still possesses fifty per cent. or more of its original inhibitory power, and since sarkosin has no inhibitory influence, we may assume that 50 per cent. of it is still present as kreatin. In boiling urine with Fehling's solution, any kreatin originally present as such, or formed from kreatinin, probably does not undergo any appreciable change in the short time during which the mixture is usually heated.

#### AN EXPLANATION OF THE REDUCING ACTION OF NORMAL URINE ON PROLONGED BOILING WITH FEHLING'S SOLUTION

As previously stated,<sup>1</sup> even normal urines give a very distinct reaction with Fehling's solution if boiled long enough. Pathological urines rich in dextrose, give a marked reaction even before the boiling point is reached, while the presence of a minute amount may necessitate boiling for some time in order to obtain any indication of the presence of sugar.

The chief reducing substances present in all normal urines

1. *Bio-Chemical Journal*, Vol. I, p. 111.

are uric acid, carbohydrate material (of which the larger part seems to be dextrose), and kreatinin. Uric acid and sugar, however, differ greatly from kreatinin in regard to the time required to cause reduction. Both substances, in the percentage in which they occur in normal urine, are capable of effecting reduction almost immediately on the boiling point being reached; in the process of reduction they undergo destruction.

Kreatinin, on the other hand, reduces very slowly indeed.

When a normal urine is boiled with Fehling's solution the uric acid and sugar present almost immediately reduce their equivalent amounts of the solution; no effect is perceived, owing to the fact that the reduced suboxide is held in solution by the kreatinin;<sup>1</sup> after boiling for a very short time, the full reducing effect of both uric acid and sugar is completed. On continued boiling the kreatinin gradually causes further reduction,<sup>2</sup> at the same time becoming gradually diminished in amount;<sup>3</sup> part of it is probably converted into kreatin, while part is destroyed.

Ultimately a point is reached at which the suboxide reduced by the sugar and uric acid, added to that reduced by the kreatinin itself, is no longer capable of being held in solution by the amount of kreatinin and its derivatives actually present in the urine at that moment; at this point precipitation occurs.

Thus it will be seen that the reaction obtained from a normal urine is very similar in character to that obtained from a urine containing more than the normal amount of sugar, the chief differences being that the more sugar present the quicker the reaction occurs.

In the presence of great excess of sugar, the effect of kreatinin is, of course, quite obscured and of no practical importance. It will be seen that the influence of kreatinin on Fehling's solution is very marked and important, though somewhat different from that ascribed to it in the text books, where its action is generally considered in

1. See page 157.

2. Experiments with aqueous solutions of kreatinin in Fehling's solution show that kreatinin reduces very slowly, and in the percentage in which it is usually present in urine gives a precipitate only after prolonged boiling (say from four to six minutes).

3. See page 167.

relation to its direct reducing power. This direct action occurs but slowly, and is therefore very insignificant when Fehling's test is used in the ordinary way; its indirect inhibitory action, however, explains many points which are otherwise obscure and for which no definite explanation has been forthcoming.

### CONCLUSIONS

1. Kreatinin is the substance in normal urine which most markedly interferes with Fehling's reaction in the presence of small amounts of sugar. Kreatin, if present, has a similar, though less marked, effect.

2. Experiment proves that kreatinin directly inhibits the effect of small amounts of sugar, when boiled with Fehling's solution, by holding the reduced suboxide in solution, and not indirectly by generating ammonia; the latter is not evolved in sufficient abundance to appreciably influence the reaction.

3. The yellow precipitate obtained in many urines containing sugar in pathological quantity is probably not the cuprous hydrate as generally stated, but the ordinary cuprous oxide, the difference in colour being dependent on the state of subdivision of the precipitate; in a yellow precipitate the particles are fine, while a red one is coarsely granular.

This difference is mainly caused by kreatinin. By varying the different amounts of sugar and kreatinin, all shades of colour from greenish-yellow to red may be obtained.

4. The colour changes observed on adding sodium-nitroprusside to an alkaline solution of either kreatinin or acetone are identical—a red colour changing to yellow, this change being accelerated by heat. Acetic acid subsequently added to the cold yellow solution gives, in the presence of kreatinin, a clear or light straw-coloured liquid, whereas acetone gives a distinct red or purplish tint.

In performing Salkowski's part of the test in which acetic acid and heat gives a blue colour, it is important not to heat the alkaline



mixture of nitro-prusside before adding the acetic acid, for sodium-nitro-prusside itself, when boiled in the presence of an alkali, is capable of giving a very distinct blue or bluish-green solution on the subsequent addition of acetic acid, apart from the presence of either kreatinin or acetone.

Acetone and kreatinin behave in exactly the same way to Sal-kowski's test—after alkaline nitro-prusside of sodium, the addition of acetic acid gives on subsequent boiling a green or blue coloured fluid, from which a precipitate ultimately settles.

5. Kreatinin, when boiled with caustic alkali of half the strength in which it is present in Fehling's solution, quickly diminishes in amount; part of it is probably converted into kreatin while part is destroyed; after boiling a weak solution of kreatinin for from four to seven minutes only about 50 per cent. of it remains.

6. Kreatin resists the action of caustic alkali much longer than kreatinin.

7. Sugar is very easily acted on by caustic alkali, and in weak solutions can be absolutely destroyed by boiling with 2-4 per cent. NaOH for some seconds.

8. The influence of kreatinin, as described above, explains why normal urine reduces Fehling's solution after prolonged boiling, and clears up many reactions in which a precipitate appears after boiling for some time with Fehling's solution.

## ON ZYMOIDS

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As the result of the recent work of E. Fischer, H. E. Armstrong, E. F. Armstrong, W. M. Bayliss and others, strong evidence has been adduced in support of the view that the action of an enzyme involves the formation of an intermediate compound between the enzyme and the substrate. It appears that the reaction between enzyme and substrate proceeds in two stages, of which the first consists of the formation of the intermediate compound, while the breaking down of this compound into the products of enzyme action constitutes the second stage. If one considers the contrast between the high specificity of the enzymes towards the substrate and the similarity in the chemical changes which they bring about, the assumption that the two stages of enzyme action are related to two different parts of the enzyme molecule appears justified. Such a view would suggest that substances might possibly exist which have still retained their property of combining with the substrate without the power of destroying it. If this be so, we should expect to find that a substrate combined with a 'zymoid,' as Bayliss proposes to call such a substance, should be protected from the activity of the enzyme, provided that sufficient zymoid is present to satisfy the affinity of the substrate. While enzymes are recognised by the products of their activity, zymoids would give evidence of their existence by an inhibition of the activity of the enzyme to which they are related.

These considerations induced us to investigate whether an enzyme rendered inactive by exposure to heat would exhibit any such inhibitory influence on the activity of the unheated enzyme. When the

results of our first experiments were published (1) we were not aware of similar experiments bearing on this question. Since then our attention has been drawn to the observations of Pollack (2) and Schwarz (3), both of whom found marked inhibition to occur, if trypsin or pepsin was made to act in the presence of the heated enzyme. Both authors agree that the inhibitory substance is not destroyed by heat, that it is indiffusible, and that it exerts its influence by in some way affecting the fermentative process. Schwarz suggests it acts as a 'negative catalyst,' since no evidence could be obtained that it directly affects either the substrate or the enzyme.

Our observations, which refer to pepsin, rennin, taka-diastrase and emulsin, agree in the main point with those of Pollack and Schwarz in that the addition of heated enzyme to active enzyme produces a strong inhibition of the reaction. In many other respects, however, our investigation has led to different results, and has thrown light on the mode of action of those inhibitory substances.

#### EXPERIMENTS WITH PEPSIN

For these experiments various preparations were used, namely, (1) an infusion of pig's or rabbit's stomach, (2) Benger's liquor pepticus, (3) pepsin (powder), and (4) pepsin (scales). These two last preparations were obtained from Duncan and Flockhart. The pepsin solutions were rendered inactive (*a*) by keeping them at temperatures varying from 56° to 60° for twenty to thirty minutes, (*b*) by keeping them in a boiling water bath for an equal time. A precipitate which appeared on heating was removed by filtration; the filtrate was used for the experiments. A series of tubes was then prepared containing a constant amount of fresh active pepsin to which varying amounts of heated pepsin were added. The tubes were filled up to a constant volume with dilute HCl of the same concentration as the HCl contained in the pepsin solution. Mett's tubes were added and the mixture incubated for periods varying from eight to sixteen hours.

A typical experiment in which liquor pepticus was employed may be given here.

								Control
Pepsin	...	0.5 c.c.	...	0.5 c.c.	...	0.5 c.c.	...	0.5 c.c.
Pepsin 56°	...	0.5 "	...	1 "	...	5 "	...	10 "
HCl	...	10 "	...	9 "	...	5 "	...	10 c.c.
Units of column of digested eggwhite	...	10	...	16	...	8	...	0

It will be seen that an excess of a solution of pepsin heated to 56° produces a strong inhibition. If, however, a similar series of experiments is made with a solution of pepsin heated previously to 100° for twenty minutes, the addition of the heated enzyme does not affect the action of the fresh pepsin.

							Control
Pepsin	...	...	...	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Pepsin 100°	...	...	...	0.5 "	1 "	5 "	—
HCl	...	...	...	9.5 "	9 "	5 "	10 c.c.
Units of digested eggwhite...	13			12		14	15

These experiments have been repeated frequently. We have never failed to obtain strong inhibition with solutions of liquor pepticus rendered inactive by temperatures between 56° and 60°; while similar quantities of pepsin solutions heated to 100° failed to produce any inhibition at all, and, indeed, produced in some instances a more or less marked acceleration. Experiments with infusions of pig's stomach gave similar results.

In order to decide whether the inhibitory action was brought about by a substance specific for each species, pepsin solutions prepared from the stomach of the pig and the rabbit respectively were tested against each other. By measuring the digestive power of the two solutions and diluting the more active solution, the two solutions were brought to about the same concentration.

							Control
Pepsin Rabbit	...	...	1 C.C.	...	1 C.C.	...	1 C.C.
Pepsin Pig 60°	...	...	5 "	...	10 "	...	—
HCl	...	...	5 "	...	—	...	10 C.C.
Units of digested eggwhite	...	2	...	0	...	8	
							Control
Pepsin Pig	...	...	1 C.C.	...	1 C.C.	...	1 C.C.
Pepsin Rabbit 60°	...	...	5 "	...	10 "	...	—
HCl	...	...	5 "	...	—	...	10 C.C.
Units of digested eggwhite	...	4	...	2	...	10	



Our results agree with those of Schwarz and Pollack in showing that the inhibitory substance is not specific for each species.

Our observations on the pepsin specimens three and four (Duncan and Flockhart) which represent purer pepsin preparations, differ slightly from those detailed above. Firstly, these preparations were much more stable towards heat, so that in the case of pepsin in scales (No. 4) a solution was rendered inactive only after keeping it at  $56^{\circ}$  to  $60^{\circ}$  for sixteen hours. Secondly, the inhibition which could be obtained was much smaller; in fact, with the fourth preparation (pepsin in scales) we were unable to produce an inhibition by the enzyme inactivated at  $60^{\circ}$ , while a solution of this preparation which had been inactivated by keeping it at  $100^{\circ}$  for twenty minutes possessed strong inhibitory power.

The following examples will illustrate the behaviour of these preparations:—

Pepsin (powder) ...	...	1 c.c.	...	Control 1 c.c.
Pepsin (powder) $60^{\circ}$	...	10 "	...	—
HCl ...	...	—	...	10 "
Digested eggwhite	...	25	...	30

The addition of enzyme exposed to  $100^{\circ}$  for ten minutes not only failed to inhibit the action of the enzyme, but actually increased the digestive power.

Pepsin (powder) ...	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	Control 0.5 c.c.
Pepsin (powder) $100^{\circ}$	1 "	3 "	5 "	10 "	—
HCl ...	9 "	7 "	5 "	—	10 c.c.
Digested eggwhite	19	22	20	18	15

The behaviour of this pepsin preparation stands in striking contrast to that of the pepsin in scales.

Pepsin (scales) ...	...	1 c.c.	...	Control 1 c.c.
Pepsin (scales) $60^{\circ}$ (16 hours)	...	10 "	...	—
HCl ...	...	—	...	10 c.c.
Digested eggwhite	...	26	...	25

Pepsin (scales) ...	...	1 c.c.	...	1 c.c.	Control 1 c.c.
Pepsin (scales) $100^{\circ}$	...	10 "	...	5 "	—
HCl ...	...	—	...	5 "	10 c.c.
Digested eggwhite	...	0	...	0	26

The significance of these differences will be discussed later on.

The inhibitory substances do not easily dialyse through parchment tubes (Schleicher and Schüll's 'Diffusions-Hülsen'). After three days the dialysate did not produce any inhibition. When the process was continued for three weeks the dialysate possessed distinct inhibitory properties.

### RENNIN

The experiments with rennin were made with various rennin preparations, which were procured from neighbouring chemists. The enzyme solutions were heated in the same way as has been described for pepsin, and a similar series of experiments was arranged.

The results which we obtained at first were very irregular. In some cases a distinct inhibition was observed if rennin heated to 60° was present, while in others we failed entirely to get any evidence of an inhibitory action. It was noticed, however, that by mixing the various reagents in a definite order, so that the active rennin was added last, the results became much more regular, and inhibition was found to occur more frequently.

The following experiments, in which the rennin was added last, may serve as an example :—

				Control
Rennin ... ..	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Rennin 60° ... ..	1 "	3 "	5 "	—
Na <sub>2</sub> CO <sub>3</sub> solution ...	9 "	7 "	5 "	9 "
Milk ... ..	10 "	10 "	10 "	10 "
Time of coag. in mins.	7.5	9	10	7
				Control
Rennin ... ..	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Rennin 100° ... ..	1 c.c.	3 "	5 "	—
Na <sub>2</sub> CO <sub>3</sub> solution ...	9 "	7 "	5 "	9 "
Milk ... ..	10 "	10 "	10 "	10 "
Time of coag. in mins.	6	6	4.5	7

It will be seen that there is a distinct inhibition produced by adding an excess of rennin heated to 60°, while a solution of rennin heated to 100° is devoid of any inhibitory power; on the contrary, like pepsin, it may enhance the activity of the enzyme.

How great the difference is between adding the inhibitory substance before or after the addition of active rennin may be seen from the following experiments where two sets of four tubes each were filled with a mixture of definite amounts of  $\text{Na}_2\text{CO}_3$  solution and milk. In one set rennin was added first, and immediately afterwards the heated enzyme. In the other set the order of adding active and inactivated enzyme was reversed.

					Control
Rennin	...	...	1 c.c.	1 c.c.	1 c.c.
Rennin 60°	...	...	1 "	3 "	5 "
$\text{Na}_2\text{CO}_3$ solution	...		9 "	7 "	5 "
Milk	...	...	10 "	10 "	10 "
Time of coag. in mins.					
Active rennin added first			2'5	1'75	2
Heated rennin added first			5	7'5	9'5
					6

The difference is very distinct, even if the acceleration which was observed in the one set of experiments is neglected.<sup>1</sup>

By keeping the heated rennin in contact with the milk for some time before the addition of active rennin, the inhibition became even more marked, as will be seen from the following experiment :—

A mixture 5 c.c. milk + 5 c.c. rennin, 60°, + 5 c.c.  $\text{Na}_2\text{CO}_3$  solution, was prepared and divided into two halves. To one half 1 c.c. active rennin was added. Clotting occurred after nine minutes. The other half was left standing at room temperature for five minutes, then 1 c.c. of active rennin was added. Clotting occurred after 17'5 minutes.

No increase of the inhibition was observed if the active rennin was kept for five minutes in contact with heated rennin before the milk was added.

The inhibitory action of heated rennin solutions is, however, by no means a constant phenomenon. With some rennin preparations we have entirely failed to get any evidence of a retarded clotting of milk. These irregularities recall the behaviour of the different pepsin preparations.

<sup>1</sup> We are at present unable to give an explanation of this accelerating effect, which has been observed frequently although by no means regularly. An analogous effect was sometimes observed in the case of pepsin, when the amount of heated pepsin added was insufficient to produce inhibition.

In order to decide whether the inhibition produced by heated enzyme is a property common to most ferments, we investigated the behaviour of taka-diastase towards starch and emulsin towards lactose.

#### TAKA-DIASTASE

A preparation by Parke, Davis & Co. was used throughout. The enzyme action was measured by Croft-Hill's modification of Pavy-Fehling's method.

Taka-diastase	...	...	1 c.c.	...	1 c.c.
Taka-diastase 60°	...	...	10 „	...	—
Starch solution	...	...	5 „	...	5 „
Water	...	...	—	...	10 „

*C.c. of mixture required to reduce 10 c.c. copper solution*

Before incubation	...	...	9 c.c.	...	—
After incubation for four hours	...	...	7.2 „	...	5.9 „

Taka-diastase	...	...	2 c.c.	...	2 „
Taka-diastase 60°	...	...	10 „	...	—
Starch solution	...	...	5 „	...	5 „
Water	...	...	—	...	10 „

*C.c. of mixture required to reduce 10 c.c. copper solution*

Before incubation	...	...	19.5 c.c.	...	—
After sixteen hours incubation	...	...	6.3 „	...	3.9 „

All our experiments with this preparation of taka-diastase gave positive results.

The behaviour of taka-diastase heated to 100° was not investigated.

#### EMULSIN

A preparation by Merck was used. The activity of the enzyme was measured by the method employed for taka-diastase.

Emulsin	...	...	1 c.c.	...	1 c.c.
Emulsin 60°	...	...	10 „	...	—
Lactose solution	...	...	10 „	...	10 „
Water	...	...	—	...	10 „

*C.c. of mixture required to reduce 10 c.c. copper solution*

Before incubation	...	...	1.8 c.c.	...	—
After twenty hours incubation	...	...	1.5 „	...	0.8 „



All our experiments with emulsin gave evidence of a distinct inhibition due to the presence of heated enzyme. The inhibitory effect remained even after keeping a solution of emulsin at 100° for forty-five minutes.

Emulsin	...	...	...	1 c.c.	...	1 c.c.
Emulsin 100°	...	...	...	10 "	...	—
Lactose solution	...	...	...	10 "	...	10 "
Water	...	...	...	—	...	10 "
<i>C.c. of mixture required to reduce 10 c.c. copper solution</i>						
Before incubation	...	...	...	1.8 c.c.	...	—
After incubation	...	...	...	1.4 "	...	2.8 "

### GENERAL DISCUSSION OF OUR RESULTS

Our results show that in the case of the enzymes investigated the addition of an excess of an enzyme which has been inactivated by heat may produce a distinct inhibition of the action of the unheated enzyme. In that respect they confirm and extend the observations of Schwarz and Pollack. We find, however, that this phenomenon may be exhibited in varying degrees by different preparations of the same enzyme, so that even absolutely negative results may be obtained. We are informed, too, that independent observers have repeated the observations of Schwarz and Pollack and have failed to obtain evidence of any inhibition. Any attempt to explain the positive results must take into account these apparent failures.

A great many observations are on record dealing with substances which inhibit enzyme action by combining with the enzyme. Our observations agree with those of Schwarz and Pollack in showing that the inhibitory effect of heated enzymes is not brought about in this way, and that the effect of heated enzyme solutions is, therefore, not due to the presence of anti-enzymes. These two observers have been unable to trace this effect of heated pepsin or trypsin solutions back to an action of the heated enzyme on the substrate, and on the basis of this negative evidence they conclude that the inhibition depends on a substance acting as a 'negative catalyst.' Our experiments with rennin show that the inhibitory substances act directly on the substrate. They conform to the condition stated in the

introduction as supplying evidence for the existence of 'zymoids.' The existence of such substances has already been postulated by Korschun (4) and by Bayliss (5), but on different grounds.

The first-named author applied the method devised by Ehrlich for the standardisation of diphtheria antitoxin to rennin solutions filtered through a Berkefeld filter. By determining the minimal active dose and the dose completely neutralising anti-rennin he found that a decrease in the value of the first factor was not accompanied by a corresponding decrease in the value of the second factor—in other words that the activity of the rennin as measured by the clotting of milk was more affected than its power to combine with its antibody. These results which are analogous to those obtained by Ehrlich for simple lethal dose and  $L_0$  dose in the case of diphtheria toxin led Korschun to conclude that rennin contains substances analogous to the toxoids of diphtheria toxin. In observing the change in the conductivity when heated trypsin was allowed to act on gelatine, Bayliss found a fall in the conductivity to occur instead of the rise which takes place with normal trypsin and represents the breaking down of the intermediate compound. He interpreted this phenomenon as showing the presence of a body which retained its power of combination with the substrate while becoming comparatively inactive as regards the decomposition of the substrate.

Our results support the view of these authors, and adduce new evidence in its support. The existence of these zymoids in heated enzyme solutions may be explained by assuming that the enzyme has been converted into the zymoid, the heat having destroyed its proteoclastic power. But this view, which is the view we adopted in our first communication, does not afford an explanation of the different behaviour of different enzyme preparations. Nor does it agree with the observations of Korschun. We are, therefore, inclined to assume that the zymoids are preformed in the enzyme preparations, but that their presence is not recognised until they are unmasked by the destructive action of heat on the enzyme. Zymoids exist even if the preparations are obtained directly from the organism: they may have been formed by the cells or they may be ultimately derived

from the enzyme, which may have been converted into these bodies by agencies more subtle than sudden exposure to heat. This view does not exclude the possibility that the enzyme is converted into a zymoid by the action of heat. But the zymoids thus formed become merely superadded to those already existing. Different enzyme preparations vary in their composition, some containing more, others less zymoids. The difference in their behaviour towards heat is similar to that exhibited by different preparations of an active enzyme.

### CONCLUSIONS

Solutions of enzymes which have been heated to  $56^{\circ}$  to  $60^{\circ}$  for twenty to thirty minutes have a strongly inhibitory effect on the activity of the unheated enzyme, if a sufficiently great amount of heated enzyme is present.

The inhibition is destroyed, as a rule, by exposure to  $100^{\circ}$ ; in some cases, however, it may persist.

Different preparations of the same enzyme vary in the inhibitory power which they exhibit after exposure to heat.

The inhibition is not due to an anti-ferment. It is brought about by a reaction between the substrate and substances present in the inactivated enzyme.

These substances dialyse only very slowly through parchment. In the case of pepsin they are not specific for each species.

These facts point to the existence of zymoids, which are probably preformed in the enzyme preparations. Zymoids, like enzymes, differ in their resistance towards heat.

Different enzyme preparations vary in the amount of zymoids which they contain.

- (1) W. Cramer and A. R. Bearn, *Proc. Phys. Soc.*, June, 1906, p. 36: *Journ. of Physics*, Vol. XXXVI.
- (2) L. Pollack, *Beitraege z. chem. Physiol. u. Patbol.*, Vol. V, 1904, p. 95-112.
- (3) O. Schwarz, *Beitraege z. chem. Physiol. u. Patbol.*, Vol. VI, 1905, p. 524-542.
- (4) S. Korschun, *Zeitschrift f. Physiol. Chemie*, Vol. XXXVII, 1903, p. 366.
- (5) W. M. Bayliss, *Archives d. Sciences Biologiques*, Vol. XI (Supplement), 1904, p. 261 (St. Petersburg).

## THE COAGULATION OF BLOOD AND THE EFFECT OF CERTAIN DRUGS AND TOXINS UPON IT

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*(Received February 9th, 1907)*

One of the chief difficulties in experiments on blood coagulation time is the elimination of all variable factors affecting the result of the observations. Before it is justifiable to attribute to any particular drug or condition a definite effect on the time of coagulation it is necessary to take into consideration any possible disease, also the effect of diet and exercise.

Before investigating the influence of drugs on animals, I instituted a series of observations on the coagulability of the blood of a healthy man and found that the diurnal variations were considerable; food especially had an effect in prolonging the time of coagulation. That the condition was due to food was definitely shown as the changes did not occur during fasting.

It is, therefore, difficult to determine a normal coagulation time for a series of observations which holds good for more than a short period. If an experiment extends over twelve hours or more, variations due to food, drink or accidental circumstances will occur; for this reason in drawing conclusions from experiments only marked differences in coagulation time suffice as evidence, as smaller differences do not exclude the natural variations and experimental error.

The term 'coagulation time' requires some notice. Blood in contact with the vessel walls of the body does not clot, but may do so if the condition of these walls be modified by injury or disease. No satisfactory explanation can be given as to why blood in the living body should remain fluid and when shed it should coagulate, but it is known that the more nearly the conditions resemble those obtaining when the blood is in the circulation the longer will the clotting be delayed; thus blood can be kept fluid for a long time in a piece of blood vessel ligatured at both ends and which has been recently



removed from the living body. Contact with a foreign body hastens coagulation, and the larger the relative surface of the foreign body the greater will be the effect. A rise in temperature slightly above that of the normal also hastens coagulation although a low temperature retards it. Hence there is no absolute coagulation time, but only a coagulation time for each set of conditions.

In making experiments on the coagulability of the blood those observations will be the most accurate in which the physical conditions of the blood when drawn are interfered with as little as possible. If the experimental conditions are such that the normal coagulation time is very short, then it is conceivable that those conditions may partly obliterate any differences due to small variations from this normal, and although the observations may be compared relatively, still as the time is shorter the range of experimental error is increased.

The effect of a drug on the coagulation time of the blood may be either direct or indirect. Thus a drug like calcium chloride on entering the circulation may directly affect the coagulability of the blood whereas the administration of another drug may institute a series of metabolic changes which again may modify the time of coagulation. In the latter case, therefore, the effect of the drug is only indirect. The injection of  $\beta$ -naphthylamine into an animal like the rabbit leads to an elevation of the temperature, and as the result of observation I have found that the coagulation time is increased; the question then arises as to whether this increase is the result of abnormal oxidation in the tissues which may accompany the pyrexia, and therefore only indirectly dependent on the drug, or whether it is the direct effect of the drug on the blood tissue.

I give this instance as a sample of the difficulties which have to be overcome in drawing precise conclusions as to the influence of a drug on the coagulation time.

#### METHOD

Brodie and Russel's coagulometer has been employed in the great majority of the present experiments.

In addition to the precautions usually given it is necessary to

have the same number of drops of water in the central chamber during a series of observations, and each series of experiments should be undertaken by the same observer, the mean of as many observations being taken as possible.

Although there is much to recommend this method, a fallacy may occur which is difficult to remedy. When there is a considerable diminution of the surface tension of the blood, the size of the pendant drop is much altered and therefore the blood is exposed to a relatively larger surface of glass, consequently the observed coagulation time is unduly shortened. This condition was obtained in a particularly pronounced degree when experimenting with diphtheria toxin; it is, therefore, difficult to compare the later observations with the normal coagulation time taken at the beginning of the experiment. In such conditions better results may be obtained by employing Wright's method.<sup>1</sup> Under ordinary conditions Wright's method allows the blood to clot much quicker than is desirable in consequence of the relatively large area of the surface of the capillary tubes to the contained blood.

It is of interest to contrast the results obtained by the two methods, and I give below, therefore, an observation on two samples of blood drawn from a man in normal health and taken at the same time, thus :—Coagulation time taken by Brodie and Russel's instrument (mean of three experiments), three minutes ten seconds. Coagulation time taken by Wright's instrument, two minutes thirty seconds (mean of three experiments). There is a difference of forty seconds between the two determinations, and I prefer the former method because, as the time of clotting is longer, the percentage of error must be smaller.

For the purpose of these experiments rabbits were found to be by far the most suitable animals. It is easy to shave and cleanse the ear and obtain a specimen of blood by pricking the marginal vein. Drugs were injected subcutaneously with a hypodermic syringe. The experiments were so conducted that the animal received just a

1. Wright and Paramore, *Lancet*, 1905, p. 1096.

sufficiency of food during an extended experiment, and none at all in a short one of a few hours duration, and by this means the experimental error due to natural variations after food, &c., was reduced. When it was found necessary to obtain counts of the number of white corpuscles synchronously with the coagulation time, the Thoma-Zeiss method was employed; the blood issuing from a puncture of the marginal vein served for both observations.

In the experiments with  $\beta$ -naphthylamine the variations of the body temperature of the animal were taken by means of a flat-bulbed rectal thermometer, the rabbit being secured by the hind and fore-legs during the process.

### EXPERIMENTS

It is convenient first to discuss some of the results of the observations on the variations of the coagulation time in a healthy man particularly with respect to food, rest and exercise.

*Experiment I.*—Man, age 31, in normal health. The time of meals and other details are given in relation to the observations on the coagulation time.

9 a.m.	...	...	Breakfast.
11 a.m.	...	...	Coag. time, 3 minutes 10 seconds.
11 a.m. to 1.30 p.m.			Walking exercise.
1.30 p.m.	...	...	Dinner.
2.30 p.m.	...	...	Coag. time, 5 minutes 30 seconds.
3 to 5 p.m.	...	...	Walking exercise.
5.30 p.m.	...	...	One cup of tea.
7.30 p.m.	...	...	Coag. time, 3 minutes 50 seconds.
8.15 p.m.	...	...	Supper.
10 p.m.	...	...	Coag. time, 4 minutes 30 seconds.
11 p.m. to 7 a.m.	...	...	Rest in bed.
8 a.m.	...	...	Coag. time, 2 minutes 30 seconds.
(Fasting before breakfast)			

*Experiment II.*—Healthy man, age 26.

6.30 p.m.	...	...	Coag. time, 3 minutes 30 seconds.
7.15 p.m.	...	...	Dinner.
8.30 p.m.	...	...	Coag. time, 5 minutes 10 seconds

*Experiment III.*—Effect of exercise. Subject same as in Experiment I.

11 a.m.	...	...	Coag. time, 3 minutes 23 seconds.
11 a.m. to 11.20 a.m.	Fairly severe muscular exercise with dumbbells and Sandow Exerciser.		
11.25 a.m.	...	...	Coag. time, 3 minutes 10 seconds.

*Experiment IV.*

5 p.m.	...	...	Coag. time, 3 minutes 40 seconds.
5 to 5.15 p.m.	...	...	Severe muscular exercise with dumbbells.
5.20 p.m.	...	...	Coag. time, 3 minutes 25 seconds.

All these observations were made in duplicate.

On examining the observations in Experiments I and II, it is seen that the longest coagulation time occurs one hour after the principal meal of the day, the blood becoming less coagulable as absorption becomes established after the meal. Conversely, the shortest time is recorded when the subject is fasting before breakfast.

As regards the effect of exercise, Experiments III and IV show a certain amount of shortening in the time of coagulation after fifteen or twenty minutes severe physical exertion. It is possible that the specific gravity of the blood may be slightly increased after loss of fluid from the plasma of perspiration, but this was not very great during the experiments and, therefore, it is probable that the slight increase in coagulability was independent of any alteration of the specific gravity. Food and drink, therefore, are the more potent factors in modifying the time of coagulation of the blood. These experiments are typical examples taken from a large series.

The different experiments on animals are classed together in groups, and the conclusions which have been arrived at in each group of experiments are discussed separately.

*Group I.* Deals with the action of calcium chloride, citric acid and milk on the coagulation time of the blood.

*Group II.* Includes drugs which are capable of producing leucocytosis after hypodermic injection, such as colchicine and nuclein; the effect of such drugs on the coagulation time are contrasted with the effect of certain pathological conditions accompanied by leucocytosis such as catarrhal pneumonia and appendicitis.

*Group III.* Deals with agents which dilute the blood, such as normal saline solution.



*Group IV.* Deals with the effect of  $\beta$ -naphthylamine and the influence of temperature.

*Group V.* Includes experiments with diphtheria toxin.

# GROUP I

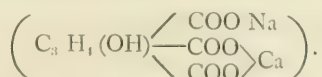
*Experiment I.*—(Citric acid). Weight of rabbit, 3·2 kilos. Normal coagulation time taken at the beginning of the experiment 2 minutes 35 seconds. '5 gram. of citric acid was injected subcutaneously at 4 p.m.

	Time of observation			Coagulation time
Commencement	4 p.m.	...	...	2 min. 35 secs.
	4.15 p.m.	...	...	2 min. 40 secs.
	4.40 p.m.	...	...	3 min. 5 secs.
	5.15 p.m.	...	...	4 min. 10 secs.

*Experiment II.* Weight of rabbit, 2·1 kilos. Normal coagulation time, 3 minutes. '5 grams. citric acid injected subcutaneously at 3.20 p.m.

	Time of observation			Coagulation time
Commencement	3.20 p.m.	...	...	3 mins.
	4 p.m.	...	...	3 min. 10 secs.
	4.20 p.m.	...	...	5 min. 45 secs.
	5 p.m.	...	...	5 min. 50 secs.

Both these experiments show very considerable lengthening of the coagulation time within one hour from the injection of the drug. We have reason to believe that citric acid combines with some of the calcium in the blood to form calcium citrate, if, therefore, by this means the calcium were removed from the circulation it is quite conceivable that the coagulation would be modified, but calcium citrate is soluble in the blood so that we might expect the calcium to exert its specific effect on the coagulation. The fact that this is not so may be explained by the views of Sabbatani<sup>1</sup> and C. J. Martin,<sup>2</sup> who have shown independently, and at the same time, that citrates act by removing the calcium ions and so preventing the formation of fibrin ferment. C. J. Martin has carried the matter a step further by demonstrating that this is brought about by the formation of a complex body containing the calcium



This substance ionises into (Na ion -) and (Ca Cit. ion +) so that on electrolysis the calcium passes to the anode.

1. Sabbatani, *Arch. Ital. de Biol.* XXXIX, 333, 1903.

2. Martin, C. J. A communication to the Physiol. Soc. at the Lister Instit., 1904.

Dr. Dixon has given me the records of the blood pressure of a cat ; the animal was receiving intravenous injections of sodium citrate, in doses of 5 c.c. of a 5 per cent. solution, at intervals during the experiment, and the coagulation time of the blood was being determined about seven minutes after each fresh injection. It was found that although the coagulability of the blood was at first decreased, as when the drug is given under the skin, yet ultimately, after continued injections of sodium citrate, a reverse effect was produced, the blood taking as long as seven and ten minutes to clot.

*Experiment III.*—Weight of rabbit, 3·2 kilos. Normal coagulation time, 3 minutes 5 seconds. '1 gram calcium chloride in 1 c.c. normal saline injected, subcutaneously, at 3.55 p.m.

Time of observation				Coagulation time
Commencement	3.55 p.m.	...	...	3 min. 5 secs.
	4.25 p.m.	...	...	1 min. 45 secs.
	4.55 p.m.	...	...	1 min. 15 secs.

*Experiment IV.*—Weight of rabbit, 2·6 kilos. Normal coagulation time, 3 min. 10 secs. '1 gram of calcium chloride injected at 12.5 p.m.

Time of observation				Coagulation time
Commencement	12.5 p.m.	...	...	3 min. 10 secs.
	12.35 p.m.	...	...	2 min. 5 secs.
	1 p.m.	...	...	1 min. 55 secs.
	1.15 p.m.	...	...	1 min. 20 secs.
	3.0 p.m.	...	...	1 min. 15 secs.

The coagulation time is very much reduced in the short space of one hour and, as seen in the last experiment, the condition remains constant after three hours.

In order to contrast the effect of calcium chloride on the coagulation time when injected hypodermically, with the effect produced when given by the mouth, '5 gram  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$  was dissolved in 5 c.c. of water and the whole solution absorbed by soft bread. This was given to a fasting rabbit and entirely devoured, the whole of the  $\text{CaCl}_2$  being thus ingested. The following two protocols show the results :—

*Experiment II' (a).*—Weight of rabbit, 2·8 kilos. Normal coagulation time, 3 mins., 5 secs. .5 gram. of calcium chloride given by the mouth at 2 p.m.

	Time of observation			Coagulation time	
Commencement	2 p.m.	...	...	3 min.	5 secs.
	2.30 p.m.	...	...	3 min.	10 secs.
	3.30 p.m.	...	...	2 min.	55 secs.
	4.30 p.m.	...	...	2 min.	58 secs.
	5.30 p.m.	...	...	2 min.	55 secs.

*Experiment II' (b).*—Weight of rabbit, 2·6 kilos. Normal coagulation time, 2 min. 50 secs. .5 gram. of calcium chloride given by mouth at 6 p.m.

	Time of observation			Coagulation time	
Commencement	6 p.m.	...	...	2 min.	50 secs.
	6.30 p.m.	...	...	2 min.	55 secs.
	7 p.m.	...	...	2 min.	52 secs.
	8 p.m.	...	...	2 min.	45 secs.
	10 p.m.	...	...	2 min.	45 secs.

On examining the last two experiments it is obvious that the time of coagulation is not appreciably affected four hours after the .5 gram. of calcium chloride had been given by the mouth, whereas .1 gram. given hypodermically very considerably shortened the coagulation time within less than an hour from the administration. I am, therefore, driven to conclude that absorption of the calcium from the stomach and intestinal canal is either not appreciable or much delayed. If this be so, it is clearly of little use to expect any considerable alteration of the coagulability of the blood in man after the administration of the drug by the mouth in ordinary doses.

Before leaving the subject of calcium chloride, it is necessary to add a word of warning on the hypodermic injection of the drug. Recently, on Dr. Dixon's suggestion, calcium chloride was injected under the skin of a patient in order to arrest an hæmoptysis which had continued for two days. The hæmorrhage stopped completely within half an hour, but a small patch of gangrene about the size of a shilling formed on the skin at the site of the injection. If calcium chloride be injected hypodermically, the solution must not be too concentrated, nor contain more than two or three grains of the salt.

As of late years many statements have appeared as to the effect of milk (indirectly through its calcium) in shortening the time of

clotting of the blood, some experiments were performed to test the validity of the statements.

*Experiment V.*—Weight of rabbit, 1·18 kilos. Normal coagulation time, 3 min. 10 secs. Ten cubic centimetres of milk were injected subcutaneously at 4.30 p.m.

Time of observation				Coagulation time
Commencement	4.30 p.m.	...	...	3 min. 10 secs.
	5 p.m.	...	...	3 min. 5 secs.
	5.30 p.m.	...	...	3 min.
	6 p.m.	...	...	3 min. 20 secs.
	9.30 a.m. (next day)			2 min. 50 secs.

*Experiment VI.*—Weight of rabbit, 3 kilos. Normal coagulation time, 3 min. 50 secs. Ten cubic centimetres of milk injected at 4 p.m.

Time of observation				Coagulation time
Commencement	4 p.m.	...	...	3 min. 50 secs.
	4.30 p.m.	...	...	3 min. 55 secs.
	5 p.m.	...	...	3 min. 30 secs.
	6 p.m.	...	...	3 min. 45 secs.

These results clearly show that milk has no decided action in increasing the coagulability of the blood. It is true that there is a slight shortening of the coagulation time at the termination of each experiment, but this is so small as to fall even within the limits of experimental error. It is also possible that, in view of the length of time occupied for the first experiment, other factors may have been introduced which would influence the later observations. It will be noticed that the weight of the rabbit used in the former experiment was 1·18 kilos.; if the weight of the blood be taken as one-thirteenth of the body weight, it may be assumed to be roughly about 81 grammes, and, therefore, its volume is about 80 cubic centimetres. Ten cubic centimetres of milk were injected, which is, therefore, a very considerable ratio when compared to the volume of the blood.

The effect of maternal milk on the coagulation time of suckling mammals was next determined.

*Experiment VII.*—A suckling rabbit was used for this experiment (weight 155 grammes). The mean of three observations gave 3 minutes 5 seconds as the coagulation time).

*Experiment VIII.*—Another suckling rabbit was taken (weight 140 grammes). The mean of three observations on the coagulation time was 2 minutes 50 seconds.



I therefore conclude that the coagulability of the blood varies but little from that of the average normal for adult rabbits.

These results on animals are not concordant with the experiments on the coagulation time of the blood of infants, as mentioned by Wright and Paramore. These observers noted the time of coagulation of the blood of six infants ranging from six weeks to six months in age; two were fed on human milk, one on human milk supplemented by cows' milk, and three on cows' milk alone. In all the six cases the maximum coagulation time did not exceed thirty-seven seconds. It must be remembered, however, that the method of capillary tubes employed by Wright and Paramore gives a relatively short coagulation time.

#### EXPERIMENTS ON THE EFFECT OF MILK DIET IN MAN

*Case I.*—M. C., woman, age 26. Health normal. The diet consisted of two pints of milk and a milk pudding for the twenty-four hours. At commencement of experiment on January 20, 6 p.m., the coag. time was 3 minutes, 10 seconds; on January 21, at 5 p.m., 3 minutes, 5 seconds.

*Case II.*—H. C., man, age 28. Health normal. Two pints of milk were added to the ordinary dietary for the twenty-four hours.

February 1.—12.30 p.m., the coag. time was 3 minutes, 25 seconds.

„ 2.—9 p.m., „ „ „ 4 „ 3 „

It does not appear from these results that milk as a diet increases the coagulability of the blood.

It is mentioned in Schafer's 'Text Book of Physiology'<sup>1</sup> that part of the calcium present in milk is united more or less firmly to the caseinogen and the excess of this base over the mineral acids to organic acids such as citric acid. The negative effect of calcium citrate on the coagulation time of the blood has already been discussed, when it was shown how it differed from salts composed of calcium and the mineral acids.

It would not, therefore, be surprising if the administration of milk produced no definite result on the coagulation time of the blood, as much of the calcium present cannot be available as a factor in controlling the coagulation.

1. Vol. I, page 130.

## GROUP II

Dixon<sup>1</sup> has shown recently that the injection of colchicine subcutaneously induces a very considerable leucocytosis, probably greater than that which can be produced by any known substance. The first effect, which lasts for about an hour after the injection of the drug, is a hypo-leucocytosis, the leucocytes being expelled from the circulation; afterwards this is followed by hyper-leucocytosis, which may attain a maximum in about twelve hours; the augmentation consists nearly entirely of the poly-morphonuclear cells. Stockman has confirmed these results in man. A similar effect, though it is slower in appearing, is obtained by the injection of small quantities of nuclein and allied bodies.

It was my purpose in the following experiments to investigate the possible influence of leucocytosis on the coagulation time of the blood, and to contrast these results with observations on clinical cases in which leucocytosis was a prominent feature, as in acute appendicitic and catarrhal pneumonia.

As a rule, no differential count of the white corpuscles has been undertaken.

*Colchicine*

*Experiment IX.*—Weight of rabbit, 2.5 kilos. Normal coagulation time, 3 minutes 30 secs. Number of white blood corpuscles per cubic millimetre, 7,200. (*Thoma-Zeiss Method.*) Two c.c. of .5 per cent. colchicine injected at 12 a.m.

	Time		Coagulation time	No. of W.B.C. per cubic millimetre
Commencement	12 a.m.	Aug. 4th	3 min. 30 secs.	7,200
	12.30 p.m.	„	3 min. 35 secs.	5,600
	1 p.m.	„	3 min. 40 secs.	3,800
	2.15 p.m.	„	3 min. 50 secs.	2,600
	3.15 p.m.	„	4 min. 30 secs.	4,160
	4.30 p.m.	„	4 min. 20 secs.	10,600
	6 p.m.	„	5 min. 0 secs.	15,000
	10 a.m.	Aug. 5th	8 min. 33 secs.	11,000
	5.15 p.m.	„	3 min. 35 secs.	5,120

(See Fig. I.)

1. *Manual of Pharmacology*, 1906.

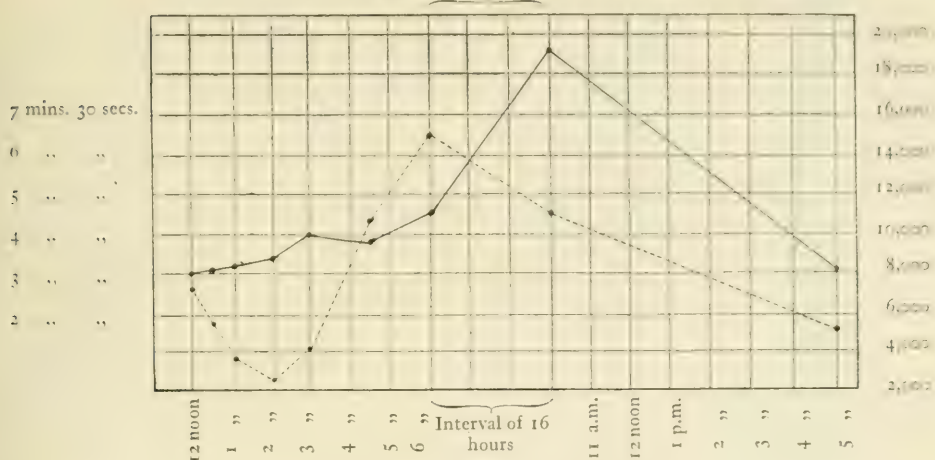


FIG. I.—EFFECT OF COLCHICINE

Two cubic centimetres of .5 per cent. Colchicine injected hypodermically at 12 noon. Black lines show coagulation time of blood. Dotted lines show number of white corpuscles per cubic millimetre.

NOTE.—The portion of the diagram included in brackets as an interval of 16 hours is not drawn to scale as regards time interval as otherwise the length of the diagram would be unwieldy for publication. The same conditions is shown in Figs. IV and V.

*Experiment X.*—Weight of rabbit, 2.7 kilos. Normal coagulation time, 3 min. 5 secs. Number of white blood corpuscles, 6,700 per cubic millimetre. Two c.c. of .5 per cent. colchicine injected at 9.30 a.m.

	Time	Coagulation time	No. of W.B.C. per cubic millimetre
Commencement	9.30 a.m.	3 min. 5 secs.	6,700
	10.30 a.m.	4 min. 20 secs.	2,900
	11.30 a.m.	3 min. 40 secs.	5,360
	12.30 p.m.	3 min. 55 secs.	8,000
	2 p.m.	4 min. 10 secs.	11,200
	3.30 p.m.	5 min. 0 secs.	16,200
	5.30 p.m.	5 min. 20 secs.	31,500
	8 p.m.	6 min. 10 secs.	31,000

(See Fig. II.)

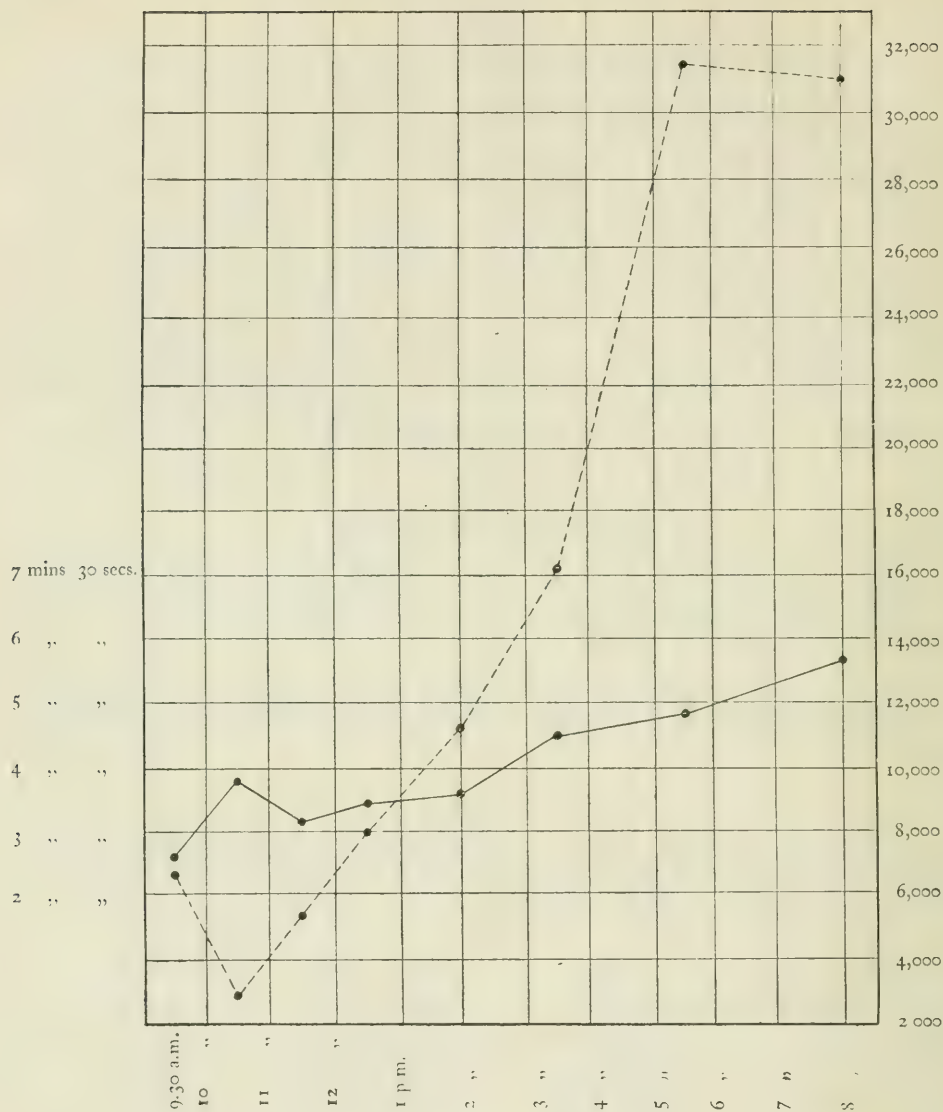


FIG. II.—EFFECT OF COLCHICINE

Two cubic centimetres of .5 per cent. Colchicine injected hypodermically at 9.30 a.m.

*Experiment XI.*—Weight of rabbit, 2.3 kilos. Normal coagulation time, 3 min. 20 secs. Number of white blood corpuscles, 7,040 per cubic millimetre. Two c.c. of .5 per cent. colchicine injected at 9.30 a.m.



	Time of observation	Coagulation time	No. of W.B.C. per cubic millimetre
Commencement	9.30 a.m.	3 min. 20 secs.	7,040
	10 a.m.	3 min. 30 secs.	5,500
	10.45 a.m.	4 min. 10 secs.	3,200
	11.30 a.m.	4 min. 30 secs.	3,584
	12.30 p.m.	5 min. 10 secs.	8,320
	2 p.m.	6 min. 0 secs.	12,800
	3.30 p.m.	9 min. 40 secs.	15,800
	5.10 p.m.	12 min. (approx.)	25,600

(See Fig. III.)

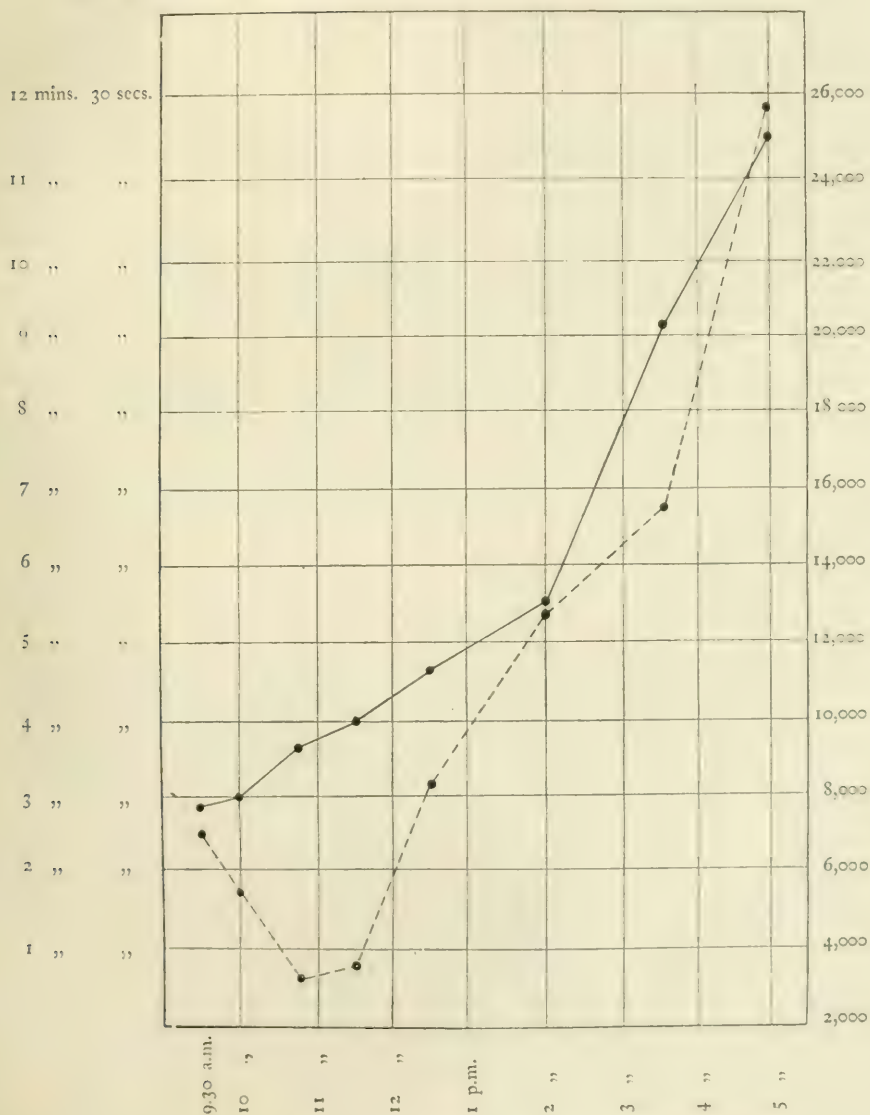


FIG. III.—EFFECT OF COLCHICINE

Two cubic centimetres of .5 per cent. Colchicine injected hypodermically at 9.30 a.m

The results of these experiments are contrasted as curves in Figures I, II and III, the black lines representing the coagulation time, and the dotted lines the number of white blood corpuscles per cubic millimetre. It is seen on examining these diagrams that during the preliminary stage, in which the number of white blood corpuscles is diminished, that the coagulation time of the blood is already somewhat lengthened. In Fig. I this is shortly followed by a reverse effect, but afterwards as leucocytosis becomes well established, in Figs. I, II and III, the coagulation time lengthens to a very considerable degree.

In Experiment XI (Fig. III), during the maximum height of the leucocytosis the coagulation time has actually reached twelve minutes; it is worth noting that in this experiment the curve representing the coagulation time ascended from the very beginning. The important point in all these experiments is the decided rise of this curve as leucocytosis increases, and it would, therefore, appear that there is some connection between them.

Although the time of coagulation increases synchronously with the leucocytosis this increase is not strictly proportional, nor do the two maxima exactly correspond. A large number of experiments would be necessary to investigate the exact relation between the two phenomena.

### *Nuclein*

When small quantities of nuclein are injected hypodermically into animals a hypo-leucocytosis occurs, which is followed by a hyper-leucocytosis. This is similar to what takes place after the injection of colchicine, except that the stage of the experiment during which the number of leucocytes is diminished is of longer duration with the nucleins. The results obtained in the two following experiments are concordant with the observations on colchicine; in both, as the time of coagulation lengthens, the leucocytosis increases.

*Experiment XII.*—Weight of rabbit, 3.1 kilos. Normal coagulation time, 4 min. 25 secs. Number of white blood corpuscles, 6,400 per cubic millimetre. Two c.c. of .5 per cent. nuclein injected subcutaneously at 1 p.m.

	Time	Coagulation time	No. of W.B.C. per cubic millimetre
Commencement	1 p.m.	4 min. 25 secs.	6,400
	2 p.m.	2 min. 10 secs.	3,200
	3 p.m.	2 min. 20 secs.	2,560
	4.15 p.m.	3 min. 20 secs.	4,480
	5.45 p.m.	3 min. 50 secs.	6,600
	9.15 a.m.	6 min. 30 secs.	16,200
	11 a.m.	6 min. 20 secs.	15,100

(See Fig. IV.)

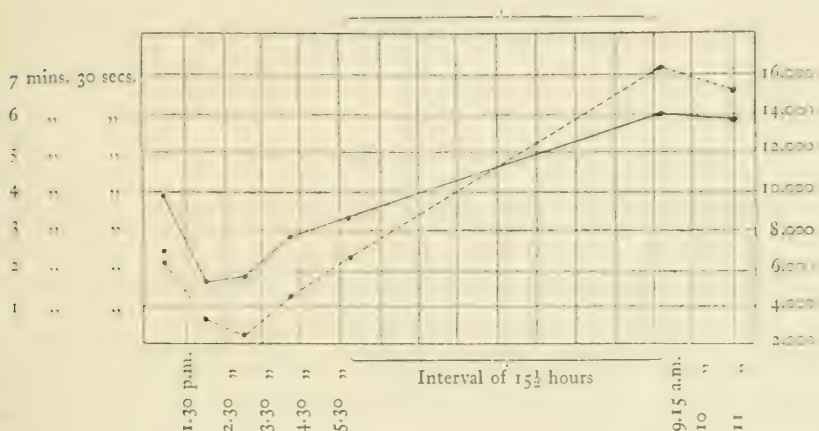


FIG. IV.—EFFECT OF 5 PER CENT. NUCLEIN

Two cubic centimetres of 5 per cent. Nuclein injected hypodermically at 1 p.m.

Note the very considerable shortening of the coagulation time during the first few hours: this was not a feature in the following experiment.

*Experiment XIII.*—Weight of rabbit, 2.5 kilos. Normal coagulation time, 3 min. 10 secs. Number of white blood corpuscles per cubic millimetre, 8,000. Two c.c. of .5 per cent. nuclein injected at 9.45 a.m.

	Time	Coagulation time	No. of W.B.C. per cubic millimetre
Commencement	9.45 a.m.	3 min. 10 secs.	8,000
	11 a.m.	3 min. 0 secs.	4,000
	12.30 p.m.	4 min. 20 secs.	3,200
	2 p.m.	6 min. 0 secs.	8,960
	4.30 p.m.	7 min. 10 secs.	10,100
	6 p.m.	9 min. 0 secs.	14,000
	9.30 a.m.	7 min. 0 secs.	12,000

(See Fig. V.)

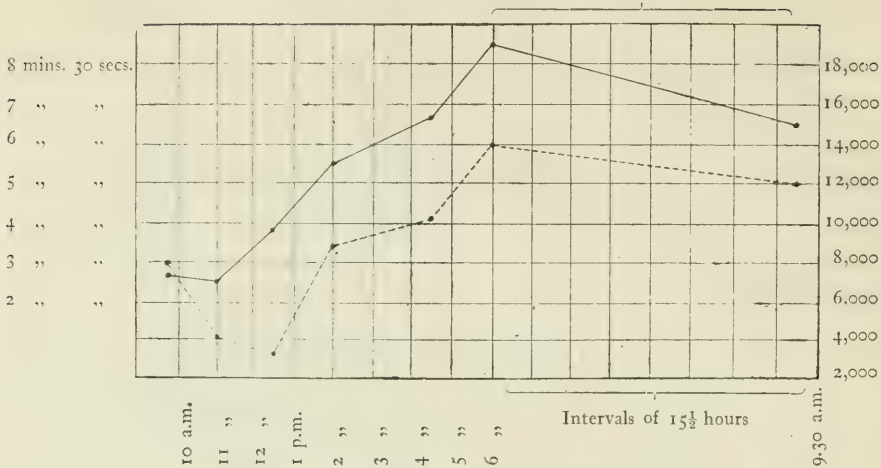


FIG. V.—EFFECT OF 5 PER CENT. NUCLEIN

Two cubic centimetres of 5 per cent. Nuclein injected hypodermically at 9.45 a.m.

I propose to contrast these experiments on animals with observations on the coagulation time of a few clinical cases of pneumonia and appendicitis in which a considerable leucocytosis was present. An increase in the number of leucocytes is nearly always one of the pathological phenomena of pneumonia. Osler, in his 'Practice of Medicine,'<sup>1</sup> points out that in most cases of pneumonia a leucocytosis appears early, persists, and ultimately disappears with the crisis, the number of leucocytes varying from 12,000 to 40,000, and even 100,000 per cubic millimetre.

### *Acute Pneumonia*

*Case I.*—T. F. P., man, age 45. Third day of disease. Respirations, 40 per minute. Temperature, 103'4. Leucocyte count shows 15,600 white blood corpuscles per cubic millimetre. Coagulation time, 6 mins. 10 secs.

*Case II.*—F. M., man, age 34. Fourth day of disease. Respiration, 35 per minute; some dyspnoea. Temperature, 102'8. Leucocytes, 25,000 per c.mm. Coagulation time, 6 mins. 30 secs.

*Case III.*—J. K., woman, age 28. Second day of disease. Respiration, 38 per minute; fine crepitations over right base. Temperature, 104'2. Leucocytes, 18,000 per c.mm. Coagulation time, 5 mins. 40 secs.



*Appendicitis*

*Case I.*—J. P., boy, age 15. Illness began six days ago. Vomiting frequent until to-day, when it ceased. Great tenderness over lower part of abdomen. Localised dulness and swelling in right iliac fossa. Constipation during last three days. Tongue dry and furred. Temperature, 101·8 F. Leucocytes, 15,600 W.B.C. per c.mm. Coagulation time, 6 mins. 15 secs. An appendical abscess was opened the day following.

*Case II.*—S. W., woman, age 23. Temperature, 102·3. Clinical signs of appendical abscess. Leucocytosis, 17,600 W.B.C. per c.mm. Coagulation time, 5 mins. 45 secs. An abscess was opened on the same day.

*Case III.*—E. C., man, age 33. Third day of present illness. Bowels confined. Tongue furred. Vomited twice yesterday. Tenderness in right iliac fossa and also near umbilicus. No obvious swelling or localised dulness. Temperature, 99·2. Leucocytosis, 15,600 per c.mm. Coagulation time, 5 mins. 30 secs. An operation was undertaken the same day the appendix was covered with inflammatory lymph and had perforated at the apex. The feature in this case was the asthenic type of the disease, although there was a definite leucocytic reaction.

These observations, as far as they go, certainly show a coagulation time which is longer than the normal average, and are, therefore, not discordant with my experiments on animals as regards a connection between leucocytosis and a decrease in the coagulability of the blood. Pye-Smith, in his article on pneumonia appearing in 'Allbutt's System of Medicine' (Vol. V), calls attention to the fact that the blood in pneumonia clots slowly, the red corpuscles subsiding before they are entangled in the meshes of fibrin; he mentions also that this condition is present in many other inflammatory diseases. Muir, in his contribution on Leucocythaemia in the same volume, points out that the blood in this disease usually coagulates less readily than normal blood.

## GROUP III

In the following experiments the normal saline solution injected subcutaneously into rabbits was found to be rapidly absorbed, and to account for a great increase in the length of the time of coagulation.

*Experiment XIV.*—Weight of rabbit, 2.5 kilos. Normal coagulation time, 3 mins. 10 secs. Eighty cubic centimetres of normal saline at 39° C. were injected subcutaneously at 3.30 p.m.

	Time of observation		Coagulation time
Commencement	3.30 p.m.	...	3 min. 10 secs.
	4 p.m.	...	10 min.
	4.30 p.m.	...	7 min.

*Experiment XV.*—Weight of rabbit, 2.1 kilos. Normal coagulation time, 3 min. 20 secs. One hundred cubic centimetres of normal saline at 39° C. were injected at 9.30 a.m.

	Time of observation		Coagulation time
Commencement	9.30 a.m.	...	3 min. 20 secs.
	10 a.m.	...	7 min. 10 secs.
	10.30 a.m.	...	8 min. 30 secs.
	11 a.m.	...	7 min. 30 secs.

The result of the two experiments are quite concordant. It is important to realise the effect of the injection of normal saline on the coagulation time, as this is a frequent procedure during severe operations or after great loss of blood.

#### GROUP IV

The administration of  $\beta$ -naphthylamine has been shown by Stern,<sup>1</sup> and afterwards by Fawcett and White<sup>2</sup>, to raise the temperature of such animals as the dog, cat and rabbit. Dixon<sup>3</sup> has verified these results. The subcutaneous injection of the drug produces great muscular weakness, and the respirations become shallow and frequent; the latter effect is especially seen in the cat. The rise of temperature may be from 3° to 4° C., although it does not invariably occur.<sup>4</sup>

It was suggested to me that an artificial febrile condition so induced might show the effects on the coagulability of the blood. It is true that objection can be brought forward that any effect on

1. Stern, *Virchow's Arch.*, CXV, p. 34, and CXXI, p. 376.

2. Fawcett and Hale White, *Journ. Physiol.*, XXI, p. 435.

3. Dixon, in Hale White's *Textbook of Pharmacology*, p. 745.

4. Dixon, *Ibid.*

the blood is not necessarily due to the pyrexia, but may depend directly on the drug. This point will be discussed later. The result of three experiments on rabbits showed that the temperature was raised about 2° C. in two cases, and in these the coagulation time became lengthened also.

The temperature was taken by a rectal thermometer.

*Experiment XVI.*—Weight of rabbit, 2.6 kilos. Normal coagulation time, 3 min. 35 secs. Temperature taken per rectum, 39.6° C. Two c.c. of 3 per cent.  $\beta$ -naphthylamine were injected at 10.45 a.m.

	Time	Coagulation time	Temperature	Remarks
Commencement	10.45 a.m.	3 min. 35 secs.	39.6	
	11.15 a.m.	3 min. 20 secs.	37.5	
	11.45 a.m.	3 min. 15 secs.	39.6	
	12.30 p.m.	3 min. 30 secs.	39.8	Respirations increased in frequency.
	2.30 p.m.	4 min. 15 secs.	40.1	
	4 p.m.	4 min. 45 secs.	41.6	Paresis of hind limbs.
	5 p.m.	5 min. 10 secs.	42.2	
	6 p.m.	5 min. 15 secs.	40.8	Great muscular weakness.

The rise of temperature was considerably delayed in this experiment, when compared with the figures given by Dixon.

*Experiment XVII.*—Weight of rabbit, 3.65 kilos. Coagulation time, 3 min. 50 secs. Temperature taken per rectum, 38.7° C. Five c.c. of 3 per cent.  $\beta$ -naphthylamine injected at 3.15 p.m.

	Time	Coagulation time	Temperature	Remarks
Commencement	3.15 p.m.	3 min. 50 secs.	38.7	
	3.45 p.m.	4 min.	38.9	
	4.15 p.m.	4 min. 30 secs.	39.4	Muscular weakness and paresis.
	5 p.m.	5 min. 10 secs.	39.8	
	5.30 p.m.	6 min. 30 secs.	41.1	A peculiar noise is occasionally made by animal.
	6 p.m.	6 min. 10 secs.	40.8	

The rise of coagulation time is coincident with the rise in temperature in both these experiments.

*Experiment XVIII.*—Weight of rabbit, 1.35 kilos. Normal coagulation time, 4 min. 15 secs. Temperature, 39.5. Three c.c. of 2 per cent.  $\beta$ -naphthylamine injected at 11.45.

	Time	Coagulation time	Temperature	Remarks
Commencement	11.45 a.m.	4 min. 15 secs.	39.5	
	12.15 p.m.	4 min.	38.9	
	12.45 p.m.	3 min. 50 secs.	38.8	
	1.15 p.m.	4 min. 10 secs.	38.8	Muscular paresis.
At 2.15, 2 c.c. of 2 per cent. $\beta$ -naphthylamine were injected in addition.				
	3 p.m.	3 min. 55 secs.	38.7	Marked paresis of hind limbs.

In Experiment XVIII, although the effect of the drug as regards muscular weakness and increased frequency of respiration was quite apparent, yet no rise in temperature was observed; on the contrary, a slight fall occurred. There was also no alteration of importance in the coagulation time. It has already been mentioned that in those experiments in which an elevation of temperature was noted the coagulation time became lengthened, whether as a result of the pyrexia or the direct effect of the drug.

As the two phenomena occurred synchronously, and were both absent from the last experiment, it is probable that there is either a connection between them or they are both the result of some common condition.

On referring to the experiments on leucocytosis and coagulation it will be noticed that the clinical cases of pneumonia and appendicitis showed varying degrees of fever.

## GROUP VI

The diphtheria toxin employed in the next experiment was of such a strength that  $\frac{1}{10}$  c.c. sufficed to kill a guinea-pig in four days.

*Experiment XIX.*—Weight of rabbit, 2 kilos. Normal coagulation time, 3 min. 10 secs. One c.c. of diphtheria toxin injected at 5 p.m.

	Time of observation			Coagulation time
Commencement	...	5 p.m.	...	3 min. 10 secs.
Next day	...	11 a.m.	...	5 min. 40 secs.

Death occurred at 12.20 a.m.

A correct determination of the coagulation time was difficult in consequence of an alteration in the viscosity of the blood. The



final observation is unduly shortened, for as the surface tension of the blood is reduced, the size of the drop is less, and the relative surface of glass to which it is exposed is greater.

#### SUMMARY

1. It is difficult to determine a normal coagulation time for a series of observations on the same animal which shall hold good for more than a short period. When an experiment is prolonged for twelve hours or more variations due to food, drink or accidental circumstances occur. For this reason, only marked differences in coagulation time are regarded as significant.

2. The administration of citric acid lengthens the time of coagulation by forming sodium citrate, a body which is incapable of ionization so as to form the Ca ion (C. J. Martin).

The hypodermic injection of calcium chloride shortens the time of coagulation. If the drug be administered by the mouth absorption is slow and incomplete, and, therefore, the effect of the drug is less.

The effect of milk, whether used as a diet or injected subcutaneously, is negative. My observations are opposed to those of Wright and Paramore.

3. When leucocytosis occurs, either as the result of some pathological condition or of the hypodermic injection of a drug, there is a lengthening of the time of coagulation of the blood.

4. Solutions of normal saline injected subcutaneously have a pronounced effect in diminishing the coagulability of the blood.

5. The experiments with  $\beta$ -naphthylamine show that after the hypodermic injection of this drug there is a lengthening of the time of coagulation.

This seems to be due to the accompanying pyrexia, because occasionally the pyretic phenomena are absent whilst the other effects of the drug on the central nervous system, heart respiration, etc., are typical; in these cases the time for coagulation remains unchanged.

6. Diphtheria toxin increases the time of coagulation.

## THE EXCRETION OF CREATININ IN A CASE OF PSEUDO-HYPERTROPHIC MUSCULAR DYSTROPHY

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(Received February 11th, 1907)

Although creatinin has long been regarded as a product of the metabolism of muscle, comparatively little information is available as to its excretion in diseases involving the muscles. In 1870 a diminution of creatinin in the urine was observed by Rosenthal in three cases of progressive muscular atrophy.<sup>1</sup> In 1877 Weiss<sup>2</sup> investigated two cases of pseudo-hypertrophic muscular dystrophy. One of these was a boy of seventeen with the disease well marked; in one day less than a decigramme, '08 gramme, of creatinin was found in the 1900 cubic centimetres of urine passed, the normal being taken at '9 gramme. The other was a strongly built male of nineteen years, less severely affected, and from the urine of a day '59 gramme of creatinin was recovered. In a third case, published in 1883, Weiss<sup>3</sup> found '63 gramme per day, the patient being a poorly nourished girl of twenty, who had suffered from the disease for four years. In 1883 Langer,<sup>4</sup> in an advanced case in a boy of eighteen with secondary lordosis, reported that the amount of creatinin never exceeded '122 gramme. Jakubowitsch,<sup>5</sup> in 1884, found a low excretion of creatinin, urea, uric acid, and

1. *Handb. d. Diagn. u. Therap. d. Nervenkrankh.* Erlangen, 1870, quoted by Langer, loc. cit. *infra*.

2. *Wien. med. Wochenschr.*, 1877, p. 701.

3. *Ibid*, 1883, p. 613.

4. *Deutsch. Arch. f. klin. Med.*, Vol. XXXII, p. 395, 1883.

5. *Diss. St. Petersburg*, 1884, Russ., reported in *Neurol. Centralbl.*, 1884, p. 279. No figures are given in the abstract

sodium chloride in two cases of the disease in children of two and a half and four years old. MacLeod,<sup>1</sup> on the contrary, in 1900, found no diminution in muscular atrophy.

In these cases the creatinin was estimated by precipitating the zinc chloride compound according to Neubauer's method, with or without Salkowski's modification. In none of them is the weight of the patient given, and, with the exception of MacLeod's cases, none are stated to have been upon a creatin-free diet. In the account of Weiss's second case it is stated that the patient took much meat, and this probably formed a part of the dietary of his third case, for the quantity of urine passed in the day was 2810 cubic centimetres; such an amount is usually associated with the metabolism of a fair quantity of proteid, and in the absence of any statement to the contrary, it is probable that some of this was taken in the form of meat.

Recent work appears to show that if creatin be given by the mouth it is not excreted in the urine as creatinin. Klercker found that the creatinin in the urine was not necessarily affected by giving meat, although the creatin was always increased in amount, and this has since been confirmed by Folin.<sup>2</sup> Meat extracts, however, contain creatinin as well as creatin, and their administration is followed by the appearance of a large proportion of the creatinin given in the urine. Since in the operations of cooking it is likely that some creatinin may be formed from the creatin in meat it is clear that any observations upon the production of creatinin in the body itself should be carried on while the patient is upon a diet containing neither meat nor its extracts.

In 1905 Folin<sup>3</sup> described a simple and rapid method of estimating creatinin by means of the colour which it strikes with picric acid in an alkaline solution, and showed that this substance is excreted in almost constant amount in the urine of any given healthy individual, the amount being independent of the total quantity of proteid metabolised. On a diet free from creatin or creatinin he found the

1. *Proc. Physiol. Soc., Journ. of Physiol.*, Vol. XXVI, p. vii, 1900.

2. *Vide Lancet*, September 15th, 1906, p. 738.

3. *Amer. Journ. of Physiol.*, Vol. XIII, p. 45, 1905.

excretion to be about 25 milligrammes per kilogramme body weight for a lean person, and 20 milligrammes for a fat one. These results have been confirmed and amplified by van Hoogenhuyze and Verploegh,<sup>1</sup> Closson,<sup>2</sup> Klercker,<sup>3</sup> and others. Closson found the daily excretion upon a vegetable diet to be 15 to 19 milligrammes per kilogramme.

The results in the cases of muscular affections above referred to were obtained for isolated days only, by the Neubauer or Neubauer-Salkowski method. Now, however, the simplicity and rapidity of Folin's method makes it easy to estimate the creatinin in the urine daily. When, therefore, a patient presented himself in the out-patient department of the hospital suffering from pseudo-hypertrophic muscular dystrophy the opportunity was taken of investigating the excretion of creatinin in this disease, and of comparing it with the published results obtained by the same method of estimation in normal individuals.

The following account of the case is founded upon the notes taken by Mr. R. F. V. Hodge, M.B., B.C., at that time House Physician at St. George's Hospital :—

The patient was a thin man of medium height, weighing 50 kilogrammes, or 7 stone 12 pounds, and by occupation a miner. His age was 29 years. His mother died of consumption when he was five years old. His three sisters and two brothers all died in infancy from causes unknown to him. The patient himself had had influenza twice, the last time six years ago. His present illness began eight years ago with pain in the back occurring in the evening occasionally after the day's work. A year later he noticed that he was getting weaker. Since then both the pain and the weakness have gradually increased, and for two years he has not been able to work. His appetite and general health in other respects have been good, but in the last six months he has lost five pounds in weight.

On admission the patient was weak but could walk. On stooping down he raised himself characteristically by 'climbing up his legs.'

The muscular system was found to be affected as follows :—The pectoralis major, especially the lower half, the trapezius, supraspinatus, latissimus dorsi, and the biceps were wasted on each side. The deltoid and the triceps were comparatively large and

1. *Zeitschr. f. physiol. Chem.*, Vol. XLVI, p. 415, 1906.

2. *Amer. Journ. of Physiol.*, Vol. XVI, p. 252, 1906.

3. *Hofmeister's Beiträge*, Vol. VIII, p. 59, 1906.



rounded, and of a harder consistency than normal resting muscle. The erector spinae, the quadratus lumborum, and the muscles of the forearm appeared normal. In the leg the thigh muscles were wasted and flabby. The anterior tibial and the calf muscles were larger and harder than normal.

Sensation was unaffected. The knee-jerks were absent. No abnormal signs were found in the thoracic and abdominal viscera.

During his stay he received a mixture containing 6 minims of liquor strychninae three times daily, and the affected muscles were galvanised and massaged. The massage was omitted, however, before the commencement of the experimental period. In two months' residence in the hospital he put on 7 pounds in weight.

For the purpose of this investigation the diet of the patient was specially controlled for ten days, and its composition calculated, the urine being collected for the same period. The patient remained in bed.

#### THE DIET

For the first four days of the experimental period, July 2nd to 5th inclusive, the patient was upon the ordinary diet of the hospital, including meat; on the 4th and 5th each article of food was weighed or measured. For the next four days, July 6th to 9th inclusive, he was placed upon a diet of starch and cream as described by Folin. This contained less than 20 grammes per day of proteid, but supplied a sufficient heat value, and enabled the excretion of creatinin to be determined with a minimum amount of proteid in the food. The source of the starch is arrowroot which is partially digested with diastase and made into a kind of blancmange, and eaten with abundance of cream, and sugar to taste. On the first day the diastatic action was too feeble and the blancmange rather hard, but afterwards it was more successful. On the fourth day, although the patient took a fair quantity, it was with but little relish, and the diet was discontinued. For the next three days the food supplied contained proteid in the form of milk and eggs, but no meat or meat extracts which would contain creatin and possibly creatinin.

In Table I the quantities of proteid, carbohydrate, and fat ingested are set forth, together with the caloric value of the food and the variations in weight of the patient.

The details of the diet for each day will be found in the appendix.

TABLE I—SHOWING WEIGHT AND DAILY FOOD

Date July, 1906	Diet	Weight kilog.	Proteid	N	Carbo- hydrate	Fat	Water	Calories per day	Calories per kilo. per day
2 3	Mixed, with meat. Not weighed	{ — —	{ — —	{ — —	{ — —	{ — —	{ — —	{ — —	{ — —
4 5	Mixed, with meat. Weighed	{ 50·3	{ 54 90	{ 8·6 15·9	{ 152 385	{ 54 67	{ 1424 1558	{ 1348 2277	{ 27 45
6 7 8 9	Starch and cream	{ 50·3 50·3 50·6 50·8	{ 19 19 19 13	{ 3·0 3·0 3·0 2·1	{ 405 405 379 277	{ 106 106 106 71	{ 2124 2124 2014 1750	{ 2726 2726 2615 1849	{ 54 54 52 36
10 11 12	Mixed with proteid. No meat	{ 50·6 50·7 50·7	{ 74 92 141	{ 11·9 14·7 22·5	{ 246 204 282	{ 121 175 223	{ 1131 2195 3290	{ 2436 2837 2803	{ 48 56 55

## APPENDIX—DIETARY

June 9th to July 3rd, inclusive. Hospital 'ordinary' diet, consisting of bread, butter, meat, potatoes, pudding, tea, soup, and a pint of milk.

July 4th. Bread 113 g., butter 28 g., pudding 113 g. (the pudding was made of 565 c.c. of milk, 57 g. of rice, and 10 g. of sugar; each pudding contained proteid 20 g., carbohydrate 78 g., and fat 16 g.), beef 113 g., potatoes 227 g., milk 565 c.c., tea 565 c.c.

July 5th. Bread 538 g., butter 28 g., pudding 113 g., mutton 113 g., potatoes 227 g., milk 565 c.c., tea 565 c.c.

July 6th. Arrowroot mixture (containing 18·2 per cent. of starch, and 77·8 per cent. of water) 1823 g., sugar 57 g., cream 425 g., salt 3 g.

July 7th. Arrowroot mixture 1823 g., sugar 57 g., cream 425 g., salt 3 g.

July 8th. Arrowroot mixture 1681 g., sugar 57 g., cream 425 g., salt 3 g.

July 9th. Arrowroot mixture 1462 g., cream 283 g., salt 3 g.

On the 6th, 7th, 8th, and 9th, 425 c.c. of water was drunk in addition to that contained in the arrowroot mixture.

July 10th. Bread 339 g., butter 57 g., milk 425 c.c., eggs 227 g., sugar 21 g., cream 142 g., Horlick's malted milk 283 c.c., water 128 c.c.

July 11th. Bread 170 g., butter 57 g., milk 425 c.c., eggs 439 g., sugar 21 g., cream 283 g., Horlick's malted milk 283 c.c., potatoes 227 g., water 847 c.c.

July 12th. Bread 170 g., butter 57 g., milk 2,000 c.c., eggs 481 g., sugar 21 g., cream 283 g., Horlick's malted milk 283 c.c., potatoes 227 g., water 565 c.c.

The food value of this dietary was calculated from published analyses for the construction of Table I. The nitrogen content of Horlick's malted milk was determined by Kjeldahl's method, from a specimen prepared as directed on the bottle, one tablespoonful being made up to half a pint with warm water. The nitrogen content of the mixture was low, being from duplicate analyses 1·82 per cent., or 1·14 per cent. of proteid.

## THE URINE

The urine was collected for the twenty-four hours from 8 a.m. to 8 a.m., for which period the diet of the corresponding day was calculated. The total nitrogen was estimated in duplicate by Kjeldahl's method, copper sulphate being used as an oxidising agent, and alizarin red as an indicator in the titration. The creatinin was estimated by Folin's method. Table II shows the figures obtained from these analyses on the different diets.

TABLE II—SHOWING TOTAL NITROGEN AND CREATININ IN THE URINE

Date July, 1906	Diet	Quantity c.c.	Total N	N per kilog.	N + —	CREATININ			
						Grammes	Creat. mg. per kilo.	Expressed as N	Creatinin N % of Total N
2	Mixed, with meat.	860	13·8	·274	—	·663	13·2	·246	1·8
3	Not weighed	1190	15·0	·297	—	·853	17·0	·317	2·1
4 <sup>1</sup>	Mixed, with meat.	(1436)	(16·3)	(·324)	—	(·930)	(18·5)	(·346)	2·1
5	Weighed	850	13·2	·262	+ 2·7	·703	14·0	·261	2·0
6	Starch and cream	530	7·2	·143	- 4·2	·511	10·2	·190	2·6
7		350	3·6	·071	- ·6	·584	11·6	·217	6·0
8		510	2·5	·049	+ ·5	·607	12·0	·226	9·1
9		510	2·0	·039	+ ·1	·566	11·1	·210	10·6
10	Mixed, with pro- teid. No meat	460	3·3	·065	+ 8·6	·454	9·0	·169	5·1
11		1190	7·8	·154	+ 6·9	·545	10·7	·202	2·6
12		2560	14·1	·279	+ 8·4	·470	9·3	·175	1·2

1. On July 4th a quantity of urine estimated by the nurse at 12 oz. was thrown away by mistake. The figures obtained for that day are calculated from the analyses of the remainder, and are included in brackets. They are evidently too high.

## RESULTS

On examining column five in Table II it is seen that the amount of creatinin passed per day upon an ordinary diet was from ·66 to ·85 grammes, while upon a creatin and creatinin free diet, such as that given from July 6th until the end of the observations, ·45 to ·6 grammes was excreted per day. In Folin's normal urines the quantity per day varied from 1·22 to 1·88 grammes, and in a man of 56·6 kilogrammes, a body weight approaching that of this patient, the amount was from 1·22 to 1·50 grammes upon a creatin free diet.<sup>1</sup> We see therefore, that the excretion of the creatinin was lower than normal

1. Loc. cit., p. 60.

though no values were obtained comparable to the extremely low ones of a decigramme, or thereabouts, found by Weiss and Langer.

The significance of the figures can be best appreciated if we consider the milligrammes of creatinin excreted for each kilogramme of body weight. The normal proportion, according to Folin, for a healthy lean man on a diet free from creatin or creatinin, is 25 milligrammes per kilogramme, or 20 for a corpulent man. Closson gives rather lower figures, 17 milligrammes per kilogramme in a man of 70 kilogrammes, and 19 in men of 57 and 61 kilogrammes, and in one subject, whose nitrogen intake was, however, exceptionally low, 15 to 16 milligrammes. In this patient, who was very thin, the excretion of creatinin upon a flesh free diet was lower than in any of the above cases, being 10 to 12 milligrammes per kilogramme on the starch and cream, and rather less, 9 to 10 milligrammes per kilogramme, on the proteid diet without meat. On an ordinary diet with meat the quantity did not exceed 13 to 17 milligrammes per kilogramme. If we take Folin's figures for a normal healthy man as a guide, we see that this patient, in proportion to his weight, was excreting less than half the normal amount. If we take Closson's estimate from three of Chittenden's subjects on a creatin free diet, 17 to 19 milligrammes per kilogramme, then this patient with 9 to 12 milligrammes was excreting less than two-thirds the normal.

Two possible explanations of this low output of creatinin suggest themselves. One is that some tissue specially connected with the metabolism of creatinin, such as muscle has been regarded, is diminished in bulk or activity, or both, relatively to the total bulk of proteid tissues. In this case the total nitrogenous metabolism would be low but the creatinin still lower, and this would be shown in the diminished percentage of the total nitrogen excreted as creatinin. Such a relative diminution of creatinin would appear to indicate that it was specially derived from the tissue known to be diminished by disease. The other possibility is that the general proteid metabolism is lessened and the creatinin proportionately with it. In this case, although the quantity would be less so would be, to an equivalent degree, the total nitrogen, and the percentage of the total nitrogen excreted as creatinin would



be the same as in a healthy person, and the conclusion would be reached that creatinin was derived from other proteid tissues to the same extent as from the wasted tissue. In columns two and three of Table II are given the total nitrogen per day and the nitrogen per kilogramme, and in the last column the percentage of the total nitrogen excreted as creatinin.

That the endogenous nitrogen excretion as a whole was diminished in this patient is shown by the figures in column two, Table II, for July 6th to 9th, when the patient was upon the starch and cream diet. On the third and fourth day of this diet, that is on July 8th and 9th, the very small quantity of 2·5 and 2·0 grammes of nitrogen was excreted, whilst Folin's subject of 55·6 kilogrammes, upon a diet identical in composition and not inferior in caloric value, excreted 5·3 and 4·4 grammes.

The four days on the low proteid diet, however, though furnishing valuable information as to the amount of 'endogenous' creatinin, do not give us the evidence we want as to the percentage of creatinin nitrogen, for this is rapidly rising owing to the fall in the so-called exogenous proteid metabolism, and, although lower to start with, by the fourth day the figures have reached the same level as in Folin's normal individuals, about 10 per cent. It was not possible to continue the diet any longer in order to see how much higher the proportion of creatinin nitrogen might have become. Also, an argument as to the source of creatinin drawn from the period during which this patient was upon such a diet might be fallacious. For in a condition of proteid starvation the tissues are not drawn upon equally to provide the nitrogen excreted, and if a creatinin furnishing tissue were called upon, and other tissues relatively spared, the percentage of creatinin nitrogen would tend to approach that of a normal individual under similar circumstances. Such a supposition would also explain why the total amount of creatinin was slightly higher on the proteid poor diet than on the liberal diet of the following three days when, as is shown in Table II, column four, nitrogen was being retained.

But when the figures from this patient upon a normal diet are

compared with those from Folin's subjects, it appears that the proportion of nitrogen excreted as creatinin is definitely diminished, as is illustrated by the following figures :—

	Folin's subjects on meat-free diet Per cent.		This patient on ordinary diet Per cent.
Proportion of total nitrogen as creatinin ...	3·2—4·5 <sup>1</sup>	...	1·8—2·1

And on the third day after the starch and cream was discontinued, the percentage of creatinin nitrogen sank as low as 1·2 per cent.

A comparison would only hold if the different individuals compared were excreting about the same amount of nitrogen per kilogramme. It is known that the quantity of proteid metabolised may vary within wide limits and is dependent in the main upon the amount in the food. If this patient were taking a great deal of proteid free from creatinin the excretion of this exogenous nitrogen would depress the proportion of creatinin nitrogen when expressed as a percentage of the whole. Column two in Table II shows that this was not the case. Both on the ordinary diet and on July 12, the third day of the proteid diet, he was not excreting more nitrogen than an ordinary individual or than men of rather greater weight, 56 kilogrammes, reported in Folin's paper.<sup>2</sup>

It appears, therefore, that in this case of pseudo-hypertrophic muscular dystrophy the creatinin excretion was diminished in greater proportion than that of the total nitrogen, and this is in harmony with the usual view that this substance is specially derived from muscle. In this disease, although some of the muscles are large, the hypertrophy is only apparent, for the actual muscular tissue is enormously diminished in amount, the bulk being made up by fatty and fibrous tissue. Even in the apparently atrophied muscles the wasting is greater than it appears for the same reason.

Creatinin has been usually regarded as a direct derivative of the creatin of muscle. Some of the facts upon which this view was founded have, however, been called into question of late, notably

1. Loc. cit., p. 62.

2. Loc. cit., pp. 60, 78.

the statement that creatin given by the mouth appears as creatinin in the urine, for Klercker, as mentioned above, states that this is not so, and has been confirmed by other workers.<sup>1</sup> Koch,<sup>2</sup> on account of the presence of the methyl group in creatinin, suggests that its likely precursors are lecithin and cephaelin in the food, though he also points out that as methylation can take place in the body, creatinin may be derived from such bodies as arginin. Gregor<sup>3</sup> concludes that it is a specific product of muscle and of other tissues. Van Hoogenhuyze and Verploegh also believe that it is formed in nonmuscular as well as in muscular tissues.<sup>4</sup>

The observations here recorded are compatible with the view that other tissues than muscle share in the production of creatinin. The association, however, of such a great diminution in the bulk of muscle fibres, and in the excretion of creatinin, together with the fact that on an ordinary diet the creatinin is also diminished relatively to the total nitrogen, appear to indicate that this substance is specially connected with the metabolism of muscle.

#### OTHER POINTS ILLUSTRATED BY THE TABLES

Table II shows well the independence of the creatinin excretion of the excretion of nitrogen, to which Folin has drawn attention. On the low proteid diet the nitrogen fell as low as 2 grammes per day, but the creatinin remained at above half a gramme. Closson<sup>5</sup> found also that there was no direct time relation between the excretion of creatinin and that of the total nitrogen, and in a recent paper Leathes<sup>6</sup> shows that upon a constant diet the rate of excretion of creatinin, like that of uric acid, is at its highest in the active hours of the day, between ten and four o'clock, sinking to a minimum at about midnight. The rate of excretion of nitrogen, on the contrary, is highest at night.

1. Czernecki, *Z. f. Physiol. Chem.*, Vol. XLIV, p. 294, 1905.
2. Koch, *Amer. Journ. of Physiol.*, Vol. XV, p. 15, 1905.
3. Gregor, *Zeitschr d. Physiol. Chem.*, Vol. XXXI, p. 98, 1900.
4. Van Hoogenhuyze and Verploegh, loc. cit.
1. Closson, loc. cit.
2. Leathes, *Journ. of Physiol.*, Vol. XXXV, p. 125, 1906.

On the low proteid diet the fall in the amount of urine to 500 cubic centimetres, or less, as shown in Table II, column one, is striking. Column six in Table I shows that the intake of fluid was rather greater than before.

The last two columns in Table I show that a high caloric value was attained with the arrowroot and cream, over 50 calories per kilogramme on the first three days, sinking on the fourth day to 36 calories per kilogramme, owing to the patient's distaste for this somewhat monotonous regimen. The body weight, as shown in column one, Table I, increased by half a kilogramme in the four days. Column four, Table II, shows that on the fourth day of the starch and cream, July 9th, the nitrogen passed out had sunk so low as to bring the patient into equilibrium. On passing to a more liberal proteid diet a large retention of nitrogen took place, amounting to 7-8 grammes, or 50 grammes of proteid, equivalent to 200 grammes of flesh per day. The quantity of urine again increased, the caloric value of the food and the patient's weight remaining about the same.

#### CONCLUSIONS

1. In a case of pseudo-hypertrophic muscular dystrophy the amount of creatinin passed out in the urine upon a diet free from meat and meat extracts was found to be about half that in normal individuals of the same weight.

2. The proportion of nitrogen excreted in the form of creatinin on an ordinary diet was less than normal.

3. Since in this case the muscular tissues had suffered much reduction, these results are in agreement with the prevailing view that creatinin is derived from muscular tissues to a greater degree than from other proteid tissues.

4. On a diet of sufficient caloric value but containing hardly any proteid, the elimination of nitrogen sank in four days to 2 grammes per day, or .039 grammes per kilogramme. Under these circumstances the proportion of nitrogen excreted as creatinin approached that found in normal individuals upon such a diet.



## A NEW CASE OF ALKAPTONURIA

BY ARCHIBALD E. GARROD, M.D., AND J. WOOD CLARKE, M.D.

*From the Laboratory for Chemical Pathology, St. Bartholomew's Hospital**(Received March 7th, 1907)*

Further records of individual cases of alkaptonuria are still to be desired as materials for the elucidation of certain points in the hereditary incidence and chemical features of this rare error of metabolism. It is on this account that notes of observations which we have recently made upon a case hitherto unrecorded are here given.

Minnie L., a remarkably fair-haired little girl, aged three years, was recently brought to the Hospital for Sick Children, Great Ormond Street, on account of the peculiar colour of her urine. There she came under the notice of Dr. Still, who, having diagnosed the case as one of alkaptonuria, kindly transferred her to Dr. Garrod's care.

The child's general health was excellent, and the only other symptom noticed besides the colour of the urine and its staining properties was occasional dysuria. This symptom was present in a case described by Stange,<sup>1</sup> and was also experienced by H. Embden<sup>2</sup> when he took a dose of eight grammes of homogentisic acid by the mouth, and so rendered himself temporarily alkaptonuric.

No history could be obtained of any similar urinary anomaly in the family of either parent. The patient is an only child, and there is no blood relationship between her parents.

The district nurse who attended to the mother at her confinement stated that she had noticed that the infant's urine stained her apron on the second day of its life. This statement is in complete accord with what was noticed in another case recorded by one of us,<sup>3</sup> in which also the staining was noticed on the second day of life.

1. *Virchow's Archiv.*, Vol. CXLVI, p. 86, 1896.

2. *Zeitschr. f. physiol. Chemie*, Vol. XVIII, p. 304, 1893.

3. *Lancet*, 1901, Vol. II, p. 1484.

The urine, which was of natural colour when passed, darkened on exposure to air, and yielded all the usual reactions of alkapton urines.

Thus the addition of a few drops of a dilute solution of ferric chloride produced a transient deep blue colour. Alkalies caused rapid darkening, especially when the liquid was warmed. The urine reduced Fehling's solution readily with the aid of heat, and the peculiar chocolate tint produced by the suspension of the orange-coloured precipitate in the dark brown liquid was characteristic.

Ammoniacal silver nitrate solution was rapidly reduced in the cold. The polarimeter showed the absence of any rotatory power of the urine.

On some days abundant uric acid crystals, tinted by the brown pigment, were spontaneously deposited.

Some of the urine was heated nearly to the boiling point, and to it was added solid neutral lead acetate in the proportion of six grammes for each 100 c.c. After filtration the clear yellow filtrate deposited, on standing, a copious crop of crystals of lead homogentisate.

Of the washed and air-dried crystals 1.6047 grammes were exposed to a temperature of  $100^{\circ}$  to  $110^{\circ}$  C. for two periods of half an hour each. The loss of weight was 0.145 gramme, which represented a loss of water of crystallisation of 9.05 per cent. The water of crystallisation in lead homogentisate  $(C_8H_7O_4)_2 Pb, 3H_2O = 9.08$  per cent.

A portion of the lead salt was finely powdered in a mortar, and was suspended in anhydrous ether through which a stream of sulphuretted hydrogen was passed. After filtration from the precipitate of lead sulphide the ether was allowed to evaporate, and in this way the free acid was obtained in colourless crystals. It melted at  $146^{\circ}$  C., which is the melting point of homogentisic acid.

Ethyl homogentisate was prepared as follows by Erich Meyer's method<sup>1</sup> :—

A portion of the free acid was dissolved in alcohol, and the solution was saturated with gaseous hydrochloric acid. After standing

1. *Deutscher Archiv. f. klin. Med.*, Vol. LXX, p. 443, 1901.

for some hours the alcoholic solution was freely diluted with water, rendered feebly alkaline with sodium carbonate, and repeatedly extracted with ether. The ethereal extract was dried over calcium chloride, and on evaporation deposited crystals of the ethyl ester, which melted at  $120^{\circ}$  C., the melting point of ethyl homogentisate.

Lastly, 500 c.c. of urine were submitted to benzoilation with benzoyl chloride and sodium hydrate. The precipitate was extracted with alcohol, and the alcohol was thrown into water, when a precipitate immediately formed. The product, once recrystallised from hot alcohol, melted at  $201^{\circ}$  C. (This agrees with the melting point of di-benzoyl homogentisamide, which is so obtained,<sup>1</sup> but after repeated recrystallisation from alcohol the melting point of that compound is raised to  $203^{\circ}$  or  $204^{\circ}$  C.)

It was thus established beyond question that the properties of the urine were due to the presence of homogentisic acid.

With a view to ascertaining whether a second alkapton acid (uroleucic acid) were also present,  $2\frac{1}{2}$  litres of the urine were subjected to Wolkow and Baumann's process<sup>2</sup> for the extraction of homogentisic acid. After acidification with sulphuric acid the urine was evaporated to a small bulk, and was thrice extracted with ether. From the collected ethereal extracts the ether was distilled off, the syrupy residue was dissolved in hot water and warmed, and a solution of basic lead acetate was freely added.

The liquid was filtered, and on standing deposited crystalline lead homogentisate. After forty-eight hours the crystals were filtered off, the excess of lead was removed from the filtrate by a stream of sulphuretted hydrogen, and the filtrate from the precipitated lead sulphide was repeatedly extracted with ether. After standing for forty-eight hours over calcium chloride to remove water and any alcohol which might be present, the ether was distilled off from the extract. A small syrupy residue which remained became crystalline on standing in a vacuum exsiccator. However, the amount of crystalline substance was very small, and we did not succeed by the

1. *Journal of Physiology*, Vol. XXVII, p. 89, 1901.

2. *Zeitschr. f. physiol. Chemie*, Vol. XV, p. 228, 1891.



use of any solvent in obtaining the crystalline substance free from the brownish syrup with which it was mixed, nor melting at so high a point as  $100^{\circ}$  C.

Two further attempts with fresh quantities of the urine were attended with no better success, and we can only state that if a second alkapton acid was present the quantity must have been extremely minute.

The child was placed upon a constant mixed diet, and after three days the urine of eight individual days was collected for the determination of the quotient  $\frac{\text{homogentisic acid.}}{\text{nitrogen.}}$

The results of these determinations, which were carried out by T. Shirley Hele and one of us, are recorded elsewhere,<sup>1</sup> and here it need only be mentioned that the quotients obtained were in accord with those got in other cases of alkaptonuria in which such determinations have been made.<sup>2</sup> They tend to confirm the conclusion that there is only one degree of alkaptonuria, and that the homogentisic acid excreted represents all the tyrosin and phenylalanin of the proteins broken down.

The average daily excretion of homogentisic acid, as determined by the silver method of Wolkow and Baumann (8 per cent. ammonia being used instead of 3 per cent.),<sup>3</sup> was 1·832 grammes.

The noteworthy points in this case are :—

1. The fact that the patient is a female, for females are few among congenital alkaptonurics.
2. The absence of consanguinity of the parents, blood relationship being common in such cases.<sup>4</sup>
3. The absence of any second alkapton acid at any rate in quantities capable of being detected by the method recommended for that purpose.
4. That the H : N quotient was in accord with those which have been obtained in other cases of alkaptonuria.

1. *Journal of Physiology*, Vol. XXXV, p. 15, Proc. Physiol. Soc., Dec. 15th, 1906.

2. *Ibid.*, Vol. XXXIII, p. 198, 1905.

3. *Ibid.*, Vol. XXXIII, p. 206, 1905.

4. *Lancet*, 1902, Vol. II, p. 1616.



# ON THE SIZE OF THE CELLS OF PLEUROCOCCLUS AND SACCHAROMYCES IN SOLUTIONS OF A NEUTRAL SALT

By ERIC DRABBLE, D.Sc., F.L.S., HILDA DRABBLE, AND DAISY G. SCOTT, M.Sc.

(Received March 7th, 1907)

In the *Bio-Chemical Journal* for February, 1906, Roaf and Whitley,<sup>1</sup> as the result of their work on tadpoles, have shown reason to believe that the size of an organism in any solution is dependent upon the osmotic concentration of that solution. The organism is believed by them to adapt itself by change of size in such a way that the difference between the osmotic concentrations inside and outside the cell remains constant. Thus, by placing tadpoles in solutions of comparatively innocuous salts, a diminution in size of the animals was observed, caused by loss of water from the constituent cells. This loss of water leads to a concentration of the cell-fluids and a consequent readjustment of balance between the internal and external pressures.

Assuming that the osmotic strength of the cell-juices in the tadpole does not differ widely from that in the adult frog, Roaf and Whitley observed that the shrinkage of the animal commenced in solutions of strength much lower than that of the cell-juices.

In the course of some work on the action of different osmotic pressures on *Pleurococcus* and Yeast we have obtained results which to a considerable degree harmonise with the views of Roaf and Whitley.

## EXPERIMENTS WITH *Pleurococcus vulgaris*, MENEGH

*Pleurococcus vulgaris*, Menegh, of normal average size was obtained from a tree trunk. This was placed in water or solutions of sodium chloride and allowed to assume a constant size.

1. Roaf and Whitley, 'The Action of acids and alkalis and of acid, alkaline and neutral salts upon the Tadpole of "Rana Temporaria."' *Bio-Chem. Jour.*, Vol. I, No. 2, 1906.

The solutions used were made up with sodium chloride for reasons stated in an earlier communication.<sup>1</sup> They were made in fractions of gram-molecular strength from 0.1 to 1.0, and the pressure of these in millimetres of mercury was calculated, due allowance being made for dissociation. It was found that from 10 to 15 minutes only were required for the cells to assume constant size and in no case during this time was the protoplasm permanently injured by the solution.

A drop of the liquid containing *Pleurococcus* was placed on a slide and the diameters of the cells were measured. Cells which were dividing or which were obviously immature as indicated by their grouping were disregarded. The diameters are given in the table in multiples of  $\mu$ , and the volumes in multiples of  $\mu^3$ . The cells were treated as spherical bodies. They depart slightly from this form, however, and this introduces a slight error but does not interfere seriously with the ratios of the volumes.

The results for *Pleurococcus* are recorded in Table I, and are plotted in the form of a curve in Fig. I.

TABLE I  
MEASUREMENT OF *Pleurococcus vulgaris*, MENEGH, IN SOLUTION  
OF SODIUM CHLORIDE

Medium	Pressure in mm. of mercury	Number of measurements made	Average diameter of <i>Pleurococcus</i> in multiples of $\mu$	Average volume in multiples of $\mu^3$
Water	—	176	15.0	1687.5
0.1 gram-molecular	3107	167	12.6	1000.2
0.2            "	6135	185	11.8	821.5
0.3            "	9076	154	11.4	740.8
0.4            "	11918	138	11.1	683.8
0.5            "	14660	134	10.9	647.5
0.6            "	17490	149	10.8	629.9
0.7            "	20253	124	10.5	578.8
0.8            "	23014	199	10.2	530.6
0.9            "	25761	211	10.1	515.1
1.0            "	28473	162	9.8	475.6

1. E. and H. Drabble. 'The Relation between the Osmotic strength of cell-sap in Plants and their Physical Environment' (*Bio-Chem. Jour.*, Vol. II, No. 3, 1907).

It will be observed that the shrinkage of the cells when placed in 0.1 gram-molecular strength of sodium chloride after being in water is very considerable, being, in fact, rather greater than that throughout the whole range from 0.1 to 1.0 gram-molecular, for whereas the diameter in water is  $15\ \mu$ , that in 0.1 gram-molecular solution is  $12.6\ \mu$  and in 1.0 is  $9.8\ \mu$ . Between the strengths 0.1 and 1.0 the successive diminutions in size are such that the curve appears to approximate to a logarithmic form.<sup>1</sup>

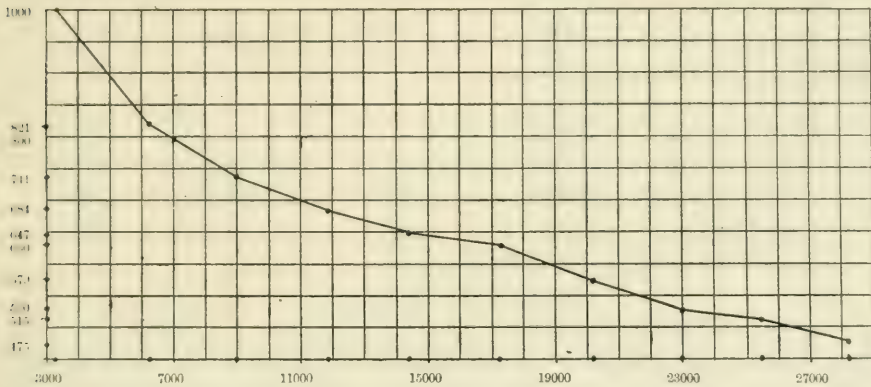


FIG. 1.—Curve expressing relation between the volume of *Pleurococcus* and the osmotic pressure of the external medium. Ordinates represent volume of the cell, abscissae represent pressure of the medium in millimetres of mercury. Volume of cell in water is  $1687\ \mu^3$ .

The cells first showed signs of plasmolysis at 0.2 gram-molecular strength, but with greater strength the protoplasm did not completely leave the wall even when the cell was placed in 1.0 gram-molecular solutions.

#### EXPERIMENTS WITH YEAST CELLS

The Yeast Cells were treated in a manner precisely similar to that described for *Pleurococcus*. Here again only mature cells were measured—any showing signs of gemmation being disregarded. The results are summarised in Table II and plotted as a curve in Fig. 2.

1. It seems probable that if the sizes in solutions of strengths between 0 and 0.1 gram-molecular had been measured, a still closer approximation to a logarithmic curve would have been found. We hope to test this point later, but at present we are only concerned with showing that the vegetable cell under certain conditions may act in a manner comparable with that of the animal cell in salt solutions.—E. D.

TABLE II

MEASUREMENT OF *Saccharomyces cerevisiae* IN SOLUTIONS OF SODIUM CHLORIDE

Medium	Pressure in mm. of mercury	Number of measurements made	Average diameter of Yeast in multiples of $\mu$	Average volume in multiples of $\mu^3$
0.1 gram-molecular	3107	16	9.5	428.7
0.2        "        "	6135	16	9.2	389.3
0.3        "        "	9076	20	8.9	352.5
0.4        "        "	11918	22	8.6	318.0
0.5        "        "	14660	30	8.3	285.9
0.6        "        "	17490	19	7.9	246.5
0.7        "        "	20253	26	7.7	228.3
0.8        "        "	23015	31	7.5	210.9
0.9        "        "	25761	31	7.1	178.9
1.0        "        "	28473	29	6.8	157.2
Yeast fluid	—	23	9.3	402.2
Actively fermenting in sugar solution	—	—	9.8	475.6

In the ordinary juice, Yeast measured  $9.3\mu$ . When placed in a 0.1 gram-molecular solution it expanded to  $9.5\mu$  while in 0.2 gram-molecular strength it became  $9.2\mu$ , i.e., a little less than its size in Yeast juice.

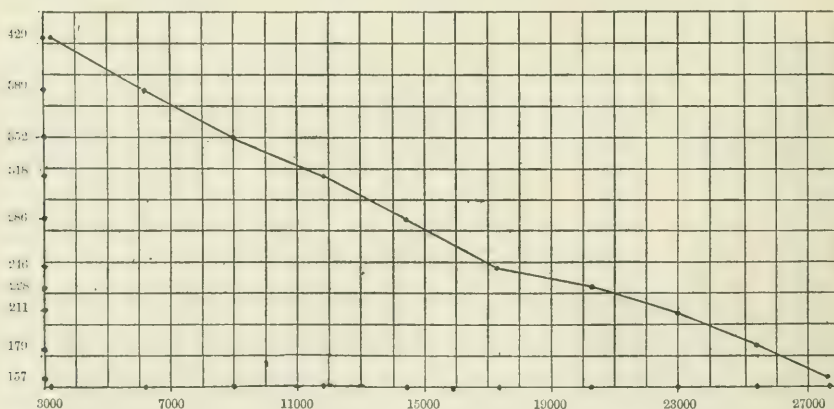


FIG. 2. Curve expressing relation between the volume of the Yeast cell and the osmotic pressure of the external medium. Ordinates represent the volume of the cell, abscissae represent pressure of the medium in millimetres of mercury. Volume of actively fermenting Yeast cell in Sugar solution =  $475.6\mu^3$ . Volume in Yeast extract =  $402.2\mu^3$ .



Another noteworthy point is that actively fermenting Yeast in sugar solution is considerably larger than resting Yeast in its own juice, having a diameter of  $9.8\ \mu$ . It is well known that sugar in solution penetrates the Yeast protoplasm readily and hence fails to exercise a lasting osmotic pressure on the cell. During fermentation, osmotically active substances are probably formed and these may serve to increase the internal pressure, leading to an expansion of the cell.

When killed in dilute hydrochloric acid, the diameter of the cell (i.e., of the external cellulose wall) is  $7.6\ \mu$ , that is, practically the same size as in  $0.8$  gram-molecular solution of sodium chloride. This suggests that the elastic rebound of the cellulose wall only comes into play in sizes greater than this, and it appears that with further shrinkage in the living cell, the wall is actually sucked in by the contracting protoplast. The question will be considered at greater length below.

#### DISCUSSION OF RESULTS

The size of an animal cell seems to be determined by a certain balance between inwardly and outwardly directed pressures. The internal outwardly directed pressure, causing expansion of the cell, is due to the osmotic activity of the internal fluids, directed against the external limiting layer of the protoplast, or against the limiting layer of the vacuole, or against both. This pressure, in a cell of constant size, must be balanced by the inwardly directed external pressure. In any liquid medium the internal pressure will cause expansion to take place until it is balanced by the external pressure counteracting it. In a naked cell this external pressure apparently resolves itself into two components; (i) the osmotic pressure of the external medium, and (ii) the resilience of the protoplast itself, that is, its resistance to stretching. These two factors taken together must balance the internal pressure. Hence, we have permanence of size taken up when the internal pressure is balanced by the external pressure *plus* the resistance to distension of the protoplast. Increase in size due to growth would be allowed for by an increase in material of the protoplast, thus allowing for further expansion before the limit of resistance is attained.

If the pressure of the external medium be altered, then supposing the resilience of the protoplast to remain unchanged, the cell will take up a new size in which again internal osmotic pressure is balanced by external osmotic pressure *plus* resilience of the protoplast. If the external medium be made of less strength than before the cell expands, if of greater strength the cell shrinks. It is, of course, possible that the alteration in the external medium may alter the resilience of the protoplast; indeed, it is highly probable, since a portion of the resilience depends on surface tension and the surface tension between the protoplast and the new strength of medium will be different from that between the protoplast and the old strength.

From what has been said already, it is perfectly evident that the cell will begin to shrink in size before the external medium becomes isosmotic with the internal sap. Indeed, shrinkage must occur with the least increase in concentration of the external medium. With a perfectly innocuous salt the shrinkage must take place through a large range of strengths, while with other salts the range will be a small one, the entrance of poisonous ions killing the protoplast and, perhaps, previously altering the penetrability of the plasmatic membrane.

In the case of plants, the course of events must be considerably modified. In the plant the plasm is surrounded by a very resistant cell-wall. Consider a vegetable cell in a medium of such strength that the plasm is kept just in contact with the cellulose wall without exerting any pressure upon it. We then have the internal osmotic pressure balanced by the external osmotic pressure *plus* the resilience of the plasmatic membrane. Now let the cell be placed in a solution of less concentration. The difference between external and internal osmotic pressures will be changed and the internal sap will exert an osmotic pressure on the wall of the protoplast which will not be balanced by the external osmotic pressure *plus* the resilience, and hence the cellulose wall will receive a certain pressure. The protoplast will tend to expand, but will be checked by the cellulose wall which will bear a part of the pressure. Now this state of affairs seems to represent what happens in all cells of land plants when placed in water. In the mature cells of land plants the extensibility of the

cellulose wall is generally very slight and hence decreased difference of pressure, brought about by increasing the external pressure of the medium, will cause but little, if any, decrease in the size of the cell as a whole (i.e., protoplast *plus* cellulose wall) as was noticed in an earlier communication.<sup>1</sup> When, however, the external osmotic pressure *plus* the resilience of the protoplast become equal to the internal osmotic pressure the protoplast will cease to exert any pressure on the cellulose wall. From this point onwards the least increase in concentration of the external medium will bring about decrease in the size of the protoplast, that is to say, plasmolysis will result.

The free cells of *Pleurococcus* and Yeast differ in their behaviour from the mature cells of leaves, in that the cells as a whole (i.e., *plus* the cellulose wall) do alter in size with change of surrounding medium. Consider *Pleurococcus* in water. Here the internal osmotic pressure is balanced by the external osmotic pressure *plus* the resilience of the protoplasmic wall (which is not stretched to its full limit and hence does not exert full pressure), *plus* the resilience of the cellulose wall. Increase in external osmotic pressure decreases the difference between external and internal osmotic pressures and hence the effect of the pressure on the interior of the cellulose wall is diminished and the cell shrinks. Continuous increase of concentration in the external medium leads to continuous decrease in the size of the cell as a whole, until the limit is reached, beyond which the cellulose wall ceases to contract. It might have been anticipated that the protoplasm would then leave the wall and complete plasmolysis ensue, but this is not necessarily the case as will be shewn when considering the behaviour of Yeast.

Yeast is accustomed to live in a fluid of greater osmotic strength than water. In this medium it assumes a size in which the internal osmotic pressure is balanced by the external osmotic pressure *plus* the resilience of the plasmatic wall, *plus* the resilience of the cellulose wall. Now in this case it is possible to place the cell in a medium of less osmotic strength than the normal one, and when this is done the cell

1. E. and H. Drabble, *Bio-Chem. Jour.*, Vol. II, No. 3.



expands. This expansion proceeds until the internal pressure is once more balanced. By taking the Yeast through successively increasing strengths of solution the cell as a whole, that is, together with its cellulose wall, decreases in size. Now, by killing the cell with dilute hydrochloric acid a certain size of cellulose wall is obtained. Presumably this is the normal size of the cell-wall, when unstretched. But this size agrees with the size of Yeast in 0.8 gram-molecular solution of sodium chloride, while increasing the strength of the external medium up to 1.0 gram-molecular, causes progressive decrease in size of the Yeast. This may be due to the fact that the protoplasm, which in 0.8 gram-molecular solution would seem to exert no pressure on the cellulose wall, does not entirely leave the wall in 0.9 and 1.0, but, as can be readily demonstrated in many cells, remains attached to it by strands of protoplasm. Further shrinkage in the protoplast would thus result in an inward pull on the cellulose wall, leading to a decrease in diameter.

There is one point upon which we are inclined to lay considerable emphasis. In such free unicellular organisms as Yeast and *Pleurococcus*, and probably also in most animal cells, the least increase in external osmotic pressure leads to decrease in size. Hence plasmolysis fails as a ready means of determining the osmotic strength of their cell-sap. In the mature cells of multicellular land plants, however, where the extensibility of the cell-wall is generally negligible, increase in osmotic strength of the external medium fails to cause shrinkage of the cell, until the externally and internally-directed pressures are equal. From that stage onwards increase in concentration of the external medium results in plasmolysis, and hence we do actually determine the isosmotic equivalent of the cell-sap, neglecting the resilience of the plasmatic wall, which, at this stage of distension, is probably extremely small.

#### CONCLUSIONS

1. The cells of Yeast and *Pleurococcus* undergo decrease in size with increase in the external osmotic pressure of the medium in which they lie.



2. This decrease in size commences in solutions of osmotic strength considerably lower than that of the cell-sap.

3. The plasmolytic method fails as a ready means of determining the osmotic strength of the cell-sap in such unicellular organisms as Yeast and *Pleurococcus* and probably also in animal cells generally.

4. In mature cells of multicellular plants the plasmolytic method is capable of yielding reliable and approximately correct measurements of the osmotic strength of the cell-sap.

## A STUDY OF METABOLISM IN A HEALTHY VEGETARIAN

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Recent work in American laboratories especially at Yale and Middletown have tended to upset orthodox calculations regarding the minimal intake and output of nitrogen in man under physiological conditions.

Hutchison<sup>1</sup> in his recent work on Dietetics places the inferior limit of proteid absorption for a healthy adult of average weight as 100 grammes or better 125 grammes which would correspond with an intake of 16 grammes nitrogen, and if nitrogenous equilibrium be established nearly the same quantity ought to be excreted.

Chittenden's<sup>2</sup> recent book on Physiological Economy shows clearly that men of average weight can live and do active work on much less ; indeed, his results show that, without loss of weight or strength, instead of 16 grammes of nitrogen being the minimal limit one third of that amount, or rather more than 5 grammes, must be accepted.

Before Chittenden's and Atwater's results were published the following case of a healthy adult vegetarian was being observed and investigated by us. The subject of our investigation is below the average weight but still a very vigorous specimen of humanity, and our results in his case tend to show that not only the physiological needs of a human body at rest but undergoing the daily demands of average mental and physical work may be supplied on very much less than 100 grammes of proteid, taken in the form of vegetarian diet.

He had been following this régime for years previously. There was nothing whatever in his appearance or attitude to men and things to

1. *Food and Principles of Dietetics*, 1900, p. 21, et seq.

2. *Studies in Nutrition*, Chas. Scribner & Sons, New York.

brand him as a 'faddist.' Being the editor of a newspaper he took a lively interest in all that was going on. It seemed advisable to ascertain exactly how much nitrogen was being absorbed and excreted and in as far as opportunities allowed this was done pretty systematically and thoroughly during two periods of observation at an interval of two years. Each period lasted for fourteen days. During the first period the urine only was examined. The food was carefully tabulated and weighed for future investigation and a blood count taken.

During the second period after a lapse of two years without any breakdown the same dietary, or as nearly the same as possible, was employed. In this case the nitrogen was estimated not only in urine but in food and faeces too. The blood count and weight were taken at the beginning and end of the experiment.

Our method for estimating the intake of nitrogen was devised to suit the circumstances, and it has been tried and found reliable by Atwater under analogous conditions. It is not at all a trustworthy proceeding nor is it easy to estimate the nitrogen in each component of the diet. So we asked the subject of our experiment to weigh and put aside for laboratory purposes an exact counterpart of everything he ate and drank. This was accumulated for each day separately, the solids in one receptacle the fluids in another. These were then carefully secured in tin vessels and conveyed daily to the laboratory where they were first weighed. The solids were then passed through a mincing machine. The fluids were added. The whole was well pounded and mixed in a large mortar to the consistency of a porridge and from a weighed sample of this the total nitrogen of the intake could easily be ascertained.

#### EPITOME OF HISTORY

The subject of the experiments was born in July, 1866; he is 5 feet 6 inches in height, and weighs 8 stone 7 pounds; the weight has been fairly constant, having increased by a few pounds during the last twenty years.

Family history good, both parents, and seven other children all living: father has had 'suppressed gout'; mother had two attacks of rheumatic fever.

The subject had swollen finger joints in youth, and was subject occasionally to severe headaches, but otherwise healthy.

He became a vegetarian at twenty to twenty-one years of age, and swelling of joints disappeared in time. At first, he used the vegetable nitrogenous foods freely, but gradually his habits changed as he found more fruit and nuts suited him better, and he now uses legumes very occasionally—perhaps twice or thrice a month.

He has resided for six years (1892-1898) in a tropical climate, two in Ceylon, and four in Siam (Bangkok). He was in perfect health during this period, living an active life, and as a naturalist visited all sorts of places, being interested in termites, which necessitated a good deal of exercise and digging.

On return home he resided in Southport (Lancs.) where probably the hard water coupled with effects of previous tropical residence brought on the only return of gouty symptoms, rheumatic pains, in winter, in chest, arms and left foot. A change of diet to fruit, nuts and brown bread, with absolute abstention from legumes, eggs, tea and coffee, and the use of boiled water only, caused the symptoms to disappear and they have not since returned.

He is not specially athletic, but is fond of walking for study of natural history, he also cycles usually a few miles daily to or from business, and has on holidays done long runs on successive days without much fatigue.

The teeth have always been poor, mastication now better by aid of artificial set.

#### SUMMARY OF DIET

The following is an indication of the nature of the meals during the 1904 period of observation, the table gives the estimations made in the well-mixed samples of exact counterparts set aside as above described.

July 4th. *Breakfast*—Manhu bread, butter, orange marmalade, coffee, milk, sugar. *Dinner*—Green peas, new potatoes, cherry cake. *Tea*—Manhu bread, butter, cocoa. *Supper*—Shredded wheat, milk, stewed gooseberries. *Exercise*—Rode 30 miles on cycle.

July 5th. *Breakfast*—Manhu bread, butter, marmalade, coffee, milk, sugar. *Dinner*—Green peas, new potatoes, boiled black-currant pudding (pastry, half Manhu and half white flour, cocoanut fat and butter). *Tea*—Bread, butter, cake (half Manhu and half white flour, cocoanut fat and butter, sultanas, raisins), cocoa. *Supper*—Shredded wheat, milk, stewed black currants. *Exercise*—In office all day, very little exercise.

July 6th. *Breakfast*—Bread, butter, marmalade, coffee, milk, sugar. *Dinner*—Butter beans, new potatoes, olive oil, mushroom catsup, boiled black-currant pudding, custard (milk, eggs, butter, sugar). *Tea*—Bread, butter, cake, cocoa, milk, sugar. *Supper*—Shredded wheat, milk, stewed red currants. *Exercise*—About 6 miles cycling.

July 7th. *Breakfast*—Bread, butter, strawberry jam, coffee. *Dinner*—Bananas, cocoanut, custard, stewed red currants. *Tea*—Bread, butter, cake, cocoa. *Supper*—Shredded wheat, milk, stewed red currants, stewed black currants. *Exercise*—In office most of day, very little exercise.



July 8th. *Breakfast*—Manhu bread, butter, marmalade, coffee, milk, sugar. *Dinner*—Bananas, cocoanut, cherries. *Tea*—Bread, butter, cake, tea. *Supper*—Bread, butter, jam. *Exercise*—In office all day, about 2 miles cycling.

July 9th. *Breakfast*—Shredded wheat, milk, jam. *Dinner*—White bread (small roll), butter, tea (about 8 ounces). *Tea*—Manhu bread, butter, cake, tea. *Supper*—Bread, butter, jam, lemon squash (one lemon, sugar, water 10 ounces, soda water 6 ounces). *Exercise*—A good deal of walking in afternoon in town. Also about 3 miles on cycle.

July 10th. *Breakfast*—Bread, marmalade, butter, coffee. *Dinner*—Bananas, cheese, strawberries, cream, sugar. *Tea*—Bread, butter, jam, tea. *Supper*—Bread, butter, cucumber, jam, soda and milk (milk 5 ounces, soda water 5 ounces). *Exercise*—Walked about 7 miles.

August 15th. *Breakfast*—Coffee, milk, sugar, bread, butter, marmalade. *Dinner*—Bananas, cheese, curds (milk turned with rennet), stewed greengages, sugar, pears. *Tea*—Cocoa, milk, sugar, bread, butter, cake. *Supper*—Bread, butter, jam, Burgundy and soda water. *Exercise*—Not much exercise—about 2 miles cycling.

August 16th. *Breakfast*—Coffee, milk, sugar, bread, butter, marmalade. *Dinner*—Green peas, potatoes, olive oil, greengage pie. *Tea*—Cocoa, milk, sugar, bread, butter, cake. *Supper*—Shredded wheat, milk, stewed greengages, sugar. *Exercise*—Cycled about 7 miles.

August 17th. *Breakfast*—Bread, butter, marmalade, coffee, milk, sugar. *Dinner*—Banana, macaroni pudding (baked with eggs, milk, and sugar), stewed greengages. *Tea*—Cocoa, milk, sugar, bread, butter, cake. *Supper*—Bread, butter, coffee, milk, sugar, cocoanut biscuit. *Exercise*—Only exercise was walking between office and home.

August 18th. *Breakfast*—Shredded wheat, milk, stewed greengages. *Dinner*—Spanish nuts grated, bananas, greengage pie. *Tea*—Tea, bread, butter. *Supper*—Shredded wheat biscuit eaten dry. *Exercise*—Only exercise between office and home. Heavy writing all day.

August 19th. *Breakfast*—Shredded wheat, milk, greengages (stewed with sugar). *Dinner*—Bananas, grated cocoanut, milk curd and stewed greengages. *Tea*—Tea, bread, butter, cake. *Supper*—Bread, butter, strawberry jam. *Exercise*—Little exercise.

August 20th. *Breakfast*—Bread, butter, strawberry jam, cocoa, milk, sugar. *Dinner*—Spanish nuts, bananas, melon, sugar. *Tea*—Bread, butter, greengage jam, cake, cocoa. *Supper*—Shredded wheat, milk, Welsh rarebit (cheese, flour, milk, egg, mustard, salt, cayenne, butter). *Exercise*—Walking and standing about good part of the day.

August 21st. *Breakfast*—Shredded wheat, milk, stewed greengages. *Dinner*—Bananas, Spanish nuts, melon, sugar. *Tea*—Bread, butter, raspberry and red currant jam, cake, tea. *Supper*—Shredded wheat, milk, stewed greengages. *Exercise*—Little exercise, but standing all afternoon.

TABLE I

## FOURTEEN DAYS' URINE OF 1902 PERIOD

	Vol. in c.c. for 24 hours	Sp. gravity	Urea in grams for 24 hours	Uric acid in grams 24 hours	Total Nitrogen in grams 24 hours	SO <sub>3</sub> in grams for 24 hours
1	510	1011	5·61	·234	2·856	—
2	510	1021	8·78	·3004	4·712	—
3	567	1020	8·81	·2251	4·286	·673
4	510	1021	9·65	·2282	4·712	·9628
5	510	1018	8·35	·218	4·141	·9363
6	454	1012	6·03	·1277	3·035	·4676
7	397	1036	10·65	·3644	5·558	1·1036
8	425	1029	11·52	·2503	5·822	1·0064
9	794	1016	12·11	·2850	6·431	·697
10	680	1016	9·41	·2522	5·168	·6534
11	624	1017	9·51	·2040	4·742	·7388
12	510	1025	14·69	·2856	7·597	1·3278
13	567	1025	15·37	·3072	7·597	1·3233
14	510	1030	18·01	·3034	8·823	1·4676

TABLE II

ESTIMATIONS OF FOOD AND EXCRETA (URINE AND FAECES) OF 1904 PERIOD

Food Date	Gross Weight Food, grms. per 24 hrs.	Urine in faeces, grms. per 24 hrs.	Total Nitrogen		Spec. grav. of urine	Chlorides as NaCl	P <sub>2</sub> O <sub>5</sub> in urine	Ratio of Do. of NaCl to N <sub>2</sub> H <sub>4</sub>	Urea in 24 hrs. grms. per	Uric acid in grms. per 24 hrs.	Iron	
			Food	Faeces							Food	Faeces
1st 4th 11th												
Week A. 1st	1023	202	5'75	2'20	6'11	1034	—	—	12'6	3'45	—	—
" 2nd	1515	190	10'13	2'56	6'79	1019	5'23	—	11'71	2'87	—	—
" 3rd	1723	310	10'89	4'17	5'32	1030	5'85	—	10'54	2'94	—	—
" 4th	1635	265	9'58	3'18	5'78	1025	4'69	—	11'02	2'41	—	—
" 5th	1230	87	6'41	0'87	4'49	1033	3'60	—	8'50	2'26	—	—
" 6th	960	347	5'93	(lost)	4'21	1034	2'51	—	7'54	1'91	—	—
" 7th	1440	360	8'20	3'58	5'01	1033	3'16	—	9'80	2'45	—	—
			9526	1761	2730	5689	—	3771				
Ave. 18th-22nd:												
Week B. 1st	1861	276	10'63	3'47	5'85	1019	4'79	1'67	11'71	2'25	1'31	0'65
" 2nd	1887	320	11'05	3'21	6'59	1018	6'79	1'92	15'48	2'96	1'49	0'95
" 3rd	1389	55	6'69	0'91	5'79	1018	5'97	1'65	11'47	2'51	0'82	0'70
" 4th	1163	238	5'78	2'99	5'14	1015	4'62	1'35	9'88	2'46	0'45	0'14
" 5th	933	285	5'49	2'77	4'73	1031	3'07	1'56	8'29	2'68	0'49	0'18
" 6th	1426	192	11'54	2'39	6'06	1032	4'55	1'60	11'78	2'78	0'68	0'49
" 7th	1304	271	5'54	2'79	5'94	1018	3'07	1'52	11'03	2'43	0'52	0'22
			9963	1637	4290	5672	18'53	40'10				

Total food nitrogen = 107'68 grms. for 13 days.

Total (urine and faeces) nitrogen = 108'69 grms. for 13 days.

Percentage of protein not absorbed = 30'8 for 13 days.

The table (Table II) shows the total daily amount of food, the amount of nitrogen therein, the amount of nitrogen excreted in urine and faeces, as also the output of urea, uric acid, chlorides, phosphates and iron.

Attention may be drawn first to the small amount of food consumed, although there was complete nutrition, the weight in fact slightly increasing during the period of experiment. The total intake of nitrogen only amounts to slightly over 8 grammes on the average for the fortnight, corresponding to about 50 grammes of proteid, and as the excretion in the faeces amounts to about 30 per cent. it is clear that still less than 50 grammes a day is utilised by the organism.

Taking fully into account the patient's weight, the deduction may safely be drawn that for a person of similar constitutional habits but of average weight, 70 grammes of proteid, and that chiefly as vegetable proteid, will suffice for the maintenance of equilibrium. The food is obviously not all vegetable in origin, there being a fair allowance of milk and butter; still a large amount of the proteid used was of vegetable origin; and the dietary shows that health and equilibrium of nutrition can be maintained not only on much less proteid than has been until recently held possible, but also that this less supply of proteid may be given in great part in vegetable forms.

The results cast a light on the metabolism of the large races of mankind which live chiefly on rice and other carbohydrate food, and show that equilibrium under such conditions and a healthy life are quite possible. Hitherto the only possible explanation, based on the minimum demand for proteid shown by Western dietaries such as those of v. Ranke and Voit, was that such races obtained proteid in some unconsidered form in amount necessary to make up to the supposed minimal quantity. Here, however, is an undoubted case, where any fraud may be ruled out, and where samples from exact duplicates of the food were carefully analysed daily, in which the amount of daily proteid does not exceed that which would be found in the daily vegetable, chiefly carbohydrate, dietary of an Eastern race.



The output of urine was excessively low. The weight of material (water) which must have left the body by the skin and lungs is about equal to that excreted as urine and faeces together. Thus, in first week, total ingesta = 9526 grammes; faeces and urine = 4491 grammes; therefore excretion by skin and lungs = 5035 grammes; during second week, total ingesta = 9963 grammes; faeces and urine = 5927, therefore excretion by skin and lungs = 4036 grammes.

### *Intake and Output of Iron*

For the sake of completeness as well as for the interest and importance of this department of metabolism, we estimated the intake and output of iron for the last seven days.

The method employed was that recommended by Stockman,<sup>1</sup> great care being taken to entirely remove organic matter, before reducing with zinc to the ferrous condition.

If we accept Stockman's<sup>2</sup> statement the quantity of iron in ordinary dietaries seldom exceeds 10 milligrams per day, and may be as low as 6 milligrams in people of ordinary appetite and digestion, we must infer that the above vegetarian dietary provided an ample supply. The average amount of iron for the seven days' dietary estimated, reached 8 milligrams daily, and the average output in the faeces was 3 milligrams daily, which corresponds closely with the output in one of Stockman and Greig's subjects.

Since it is well known that practically no iron is excreted in the urine, these figures indicate that during this period a storage of iron in the organism was occurring. It is clear that at other periods this process must be reversed, and hence in the adult, as in the foetus, the liver and other haematopoietic organs must possess the power of storing and setting free iron as occasion requires. Several examinations of the subject's blood demonstrated that he was normal in haemoglobin and had a low average count of leucocytes. It is clear, therefore, from the point of view of low metabolism that the

1. *Journ. of Physiology*, Vol. XVIII, p. 484, 1895.

2. Stockman & Greig, *Ibid*, Vol. XXI, p. 55, 1879.

above apparently meagre diet contains sufficient ferruginous material for the wants of the body. The minute amounts involved tend to corroborate Bunge's theory that in anaemia the inorganic salts of iron when administered are not directly absorbed but react indirectly on some of the fermentative or digestive products present in the intestine, which would otherwise abstract iron from the body. A certain parallelism between retained iron and excreted nitrogen is observable in the Table, which possibly suggests that in the course of metabolism albuminised iron plays an important part.

The presence of gout in the family history and in that of the subject himself is interesting for two reasons:—

(1) Apparently this vegetarian régime had a beneficial effect in course of time in removing the deposits from the fingers.

(2) The recurrence of the arthritic symptoms during residence in a most salubrious locality, but close to the sea and with a hard water supply.

The latter observation suggested the comparison of the urinary inorganic bases with those of a case of acute podalgic gout, and this was done with a subject of about the same age and height who was an excessive meat eater. It may be added that the vegetarian consumed no table salt other than that in the food, while the meat-eater used large quantities.

The distribution of bases in the inorganic constituents of the urine was estimated for one period of twenty-four hours in each case.

The results are shown in the following statement:—

	Volume of urine	Na <sub>2</sub> O	K <sub>2</sub> O	CaO	MgO
Healthy vegetarian	250	1·520	0·543	0·039	0·139
Gouty patient	650	1·575	0·439	0·206	0·179

Stated as percentages of total base the figures become in each case :

	Na	K	Ca	Mg
Vegetarian	67·8	24·4	1·3	6·3
Gouty patient	67·5	16·8	8·4	7·1

The difference in calcium output is the most interesting fact given by the above figures, especially when it is remembered that the total amount of urine excreted by the gouty patient was more than double that of the vegetarian.

The figures suggest that perverted calcium excretion may be a factor in the pathogeny of gout, and that in gout an excess of calcium is being excreted by the kidneys, which under normal conditions would be removed by intestinal excretion.

Results which will shortly be published show that inorganic salts have a marked depressing action on the solubility of urates, and hence the vegetarian urine would be in this comparison a much more favourable vehicle than that of the gouty patient for uric acid removal.

# ERGOTOXINE AND SOME OTHER CONSTITUENTS OF ERGOT

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In papers already published by us (partly in conjunction with F. H. Carr) we have described certain physiological effects produced by ergot preparations and the isolation and chemical properties of the alkaloid ergotoxine, to which these effects are due. In the present paper<sup>1</sup> we propose to describe more completely the general physiological action of this alkaloid, and to discuss, in the light of our earlier experiments, its occurrence in the numerous substances which have from time to time been described as active principles of ergot.

1. For the chemical experiments G. Barger is responsible, for the physiological H. H. Dale.



## HISTORICAL

Though ergot occupied the attention of a number of chemists in the eighteenth century and the earlier part of the nineteenth—Vauquelin (1), Pettenkofer (2)—the first careful investigation was made by Wiggers (3) in 1831, at a time when the long controversy as to the therapeutic utility of the drug was drawing to a close. Wiggers found 35 per cent. of oil, a crystalline wax-like substance, which he termed *cerin*, and which is probably identical with the ‘*ergosterine*’ of Tanret, a new sugar, later found to be identical with *trehalose*, and phosphates. He also proved that, contrary to the statements of some of his predecessors, starch and hydrocyanic acid are absent, and described a resin, soluble in alcohol but insoluble in ether and in water, which he termed ‘*ergotin*.’ From feeding experiments on cocks he concluded that the toxic properties of ergot are wholly due to this resin. The therapeutic activity he regarded as due to a water-soluble substance, on account of the good effects of liquid (aqueous) extracts when used in medicine—an opinion which still finds many supporters.

Considering the state of chemical knowledge at the time, Wiggers’ analysis was so complete that it satisfied chemists for a generation. With the exception of Bonjean (4), who in 1842 described a method for preparing an aqueous extract, which he likewise called *ergotin*, and which has been adopted in some form or other in most pharmacopoeias, no substantial advance was made till Wenzell (5), in 1864, obtained two fixed alkaloids from ergot, ‘*ergotine*’ and ‘*ecboline*.’ This discovery led to researches by Manassewitz (6), Herrmann (7), and Ganser (8), who for the most part confirmed Wenzell’s results. The whole question, however, was still in a state of great confusion, for in 1874 Buchheim (10) attributed the activity of ergot not to any specific substance, but to the ‘putrid and septic substances’ as a whole. In striking contrast to this work is that of Tanret (12), who, a year later, made the first great step by discovering a crystalline alkaloid which he prepared in a state of undoubted purity, and named *ergotinine* (to distinguish it from the resinous ‘*ergotines*’ of his predecessors). Tanret’s alkaloid has been found by all subsequent

investigators, but has not always been properly identified. Dragendorff and Podwyssotski (14) in 1877 described as 'picrosclerotine' an alkaloid which was undoubtedly identical with ergotinine, as may be inferred from the work of Dragendorff's pupil, Blumberg (15). Other synonyms for the crystalline ergot alkaloid are : sclerocrystalline, (Podwyssotski (17), 1883) and secaline (Jacobj (37), 1897). In 1894 it was erroneously described as identical with Kobert's cornutine by Keller (32), who afterwards, however, abandoned this view.

Tanret regarded ergotinine as the therapeutically active principle, a belief which led Yvon (13) in 1877, to prepare an 'ergotin,' the first of a new type of extracts rich in alkaloids. Tanret's view as to the activity of ergotinine did not, however, meet with universal acceptance, and the search for an alkaloid as the active principle was followed by a period when most investigators looked for a water-soluble acid as the active substance. The starting point of this series of investigations is the '*ergotinum dialysatum*' of Wernich (9), a water-soluble extract prepared in 1874. In 1875 Zweifel (11) described a preparation to which the name 'ergotinic acid' was later applied. Dragendorff and Podwyssotski (14) in 1877 called a similar water-soluble principle 'sclerotinic acid,' which was further referred to by Podwyssotski (17) in 1883, and by Denzel (19) in 1884.

In 1884 Kobert (20) published an elaborate investigation of ergot. In the main his results may be said to be a combination of the 'alkaloidal' and the 'acidic' view, for, of the three active substances described by him, two are acids and one is an alkaloid. For one of the acids, which is soluble in water, he retained the old name ergotinic acid. According to Kobert, it is a nitrogenous, glucosidic substance, and is the chief constituent of the sclerotinic acid of Dragendorff and Podwyssotski. It lowers the blood pressure and paralyses the central nervous system, but does not produce gangrene ; it is without action on the uterus, and does not cause vaso-constriction. Chemically, ergotinic acid was regarded by Voswinkel (29) as a carbohydrate (mannane), but Kobert's view was upheld by his pupil Kruskal (31). Quite recently it has been described by Kraft (46) as a mixture containing, among other things, mannite and a new

crystalline acid (secale-amino-sulphonic acid). Since, however, Kobert's statement that it is therapeutically useless has not been disputed, it need not concern us further.

The other substances described by Kobert are both insoluble in water, but soluble in alcohol. They are 'sphacelinic acid' and the alkaloid 'cornutine.' According to Kobert both substances produce contractions of the uterus and act on the vaso-motor centre, causing rise of blood-pressure. In these respects relatively large doses of the acid correspond to small doses of the alkaloid. The chief points of difference are that sphacelinic acid produces gangrene, and cornutine does not, and that cornutine, in small doses, has a convulsant effect superficially similar to that of strychnine. Kobert does not claim to have isolated his substances in even an approximate state of chemical purity. The one substance claiming to be an active principle which had at that time been obtained chemically pure, namely, Tanret's crystalline ergotinine, he declared to be inactive.

Kobert's theory of the existence of two principles, sphacelinic acid and cornutine, was upheld by Bombelon (26), though this author's preparations were not examined physiologically. Kobert's results were further confirmed by his pupils; in the case of sphacelinic acid by Grünfeld (30), and in that of cornutine by Lewitsky (24). Evidence both in favour of and against the therapeutic use of cornutine was adduced by various clinical observers. (See, for instance, Erhard (22), Graefe (23), Thomson (27).) In 1889 Kobert (28) contributed to the *Real-Encyklopadie der Pharmacie* his article on ergot, in which he gave a modified method for the preparation of cornutine, and strongly recommended this alkaloid for obstetrical purposes, whereas in 1884 he inclined to favour the use of sphacelinic acid.

Keller (32) in 1894 adopted this later view of Kobert, and, further assuming that ergotinine and cornutine are the same and the only alkaloid in ergot, he based a method of assay on the determination of the total alkaloid. For this supposed one alkaloid he preferred the name cornutine 'on practical grounds.'

Later, in 1896, Keller (35) regarded cornutine as a partially decomposed ergotinine, and advanced arguments against Kobert's



view that ergotinine is inactive. These arguments are based on the activity of commercial (impure) ergotinine specimens, and prove nothing as to the activity of the chemically pure base—a point with which we shall deal in a later section of this paper.

The substance described as 'cornutin' by Keller was examined physiologically in 1902 by Santesson (39), who used a specimen obtained from Keller himself, and others prepared according to the latter's directions. In frogs, rabbits, and fowls he obtained with this preparation, in considerable doses, only a partial and feeble reproduction of some of the effects attributed by Kobert to sphacelinic acid and cornutine. A significant rise of blood-pressure was obtained only in the fowl, and the effect on pregnant rodents was not of a constant or definite nature. He concludes that this substance is not the important active principle.

Up till 1906 two further attempts to isolate the active principle were made by Jacobj (37) and by Meulenhoff (38). Both these investigators adopted as their chief criterion of activity the reaction of the cock's comb, which Kobert only obtained with sphacelinic acid. According to Jacobj the active principle is a non-nitrogenous resin, with feebly acid properties, for which he adopted the name 'sphacelotoxin,' and which he described as combined in ergot with two inert substances—(a) with 'ergochrysin,' to form the compound 'chrysotoxin'; (b) with the crystalline alkaloid 'secaline' to form the compound 'secalintoxin.' Sphacelotoxin Jacobj regarded as sphacelinic acid in a pure form. Both chrysotoxin and secalintoxin caused uterine contractions and gangrene of the cock's comb. Meulenhoff likewise concluded that the activity of ergot is due to an acid resin (Kobert's sphacelinic acid). With regard to cornutine, Meulenhoff confirmed Tanret's view that it is a decomposition product of ergotinine formed by the acid used in its extraction, and does not occur in ergot as such.

It is evident from the above account that when, a few years ago, we began to work on ergot the more recent investigators had held that the activity of ergot, or, at any rate, the production of gangrene, was determined by an acidic principle. Our own



experiments have, however, led us to believe that all these acidic preparations owed their activity to a powerfully active amorphous alkaloid, of which crystalline salts were isolated by F. H. Carr and one of us, and to which the name ergotoxine was given (45). Soon afterwards Kraft (46) described the same alkaloid under the name hydroërgotinine, regarding it as the hydrate of Tanret's ergotinine. Recently ergotoxine and some of its salts have been described in detail by Barger and Carr (51), who, from their analyses, assign to ergotoxine the formula  $C_{35}H_{41}O_6N_5$ , and to ergotinine the formula  $C_{35}H_{39}O_5N_5$ , thus establishing Kraft's view as to the relationship of the two alkaloids. Meanwhile a substance of an entirely different kind, neither acidic nor alkaloidal, was described by Vahlen (44) as the essential therapeutic principle of ergot. With the nature of this substance, to which he gave the name 'clavin,' we shall deal in a later section of the paper.

## ERGOTOXINE

### *Chemical*

The chemical description of the alkaloid ergotoxine, which has already been given elsewhere by Barger and Carr (51), may be summarised as follows:—Ergotoxine is a white amorphous powder having the composition  $C_{35}H_{41}O_6N_5$ , and melting, with decomposition, at  $162^\circ$  to  $164^\circ$ . It is freely soluble in most organic solvents, but only slightly so in ether, and is insoluble in light petroleum. It is soluble in dilute caustic soda, and is a feeble monacid base. Ergotoxine forms crystalline salts, one of the most characteristic of which is the phosphate  $C_{35}H_{41}O_6N_5 \cdot H_3PO_4 \cdot H_2O$ , forming minute needles, melting at  $186^\circ$  to  $187^\circ$ .

Barger and Carr have amended Tanret's original formula for crystalline ergotinine to  $C_{35}H_{39}O_5N_5$ . (Compare with this Tanret's recent formula,  $C_{35}H_{40}O_5N_5$  (49).) Hence it will be seen that the crystalline alkaloid is the anhydride of the amorphous, as first suggested by Kraft. Either alkaloid can be readily converted into the other. Both give the colour reaction described by Tanret and by Keller as

characteristic of ergotinine. The chief differences between the two alkaloids are that ergotinine crystallises very readily, whereas ergotoxine has so far resisted all attempts at crystallisation, and that ergotoxine is very soluble in cold alcohol while ergotinine is but slightly soluble. So far only amorphous ergotinine salts have been prepared, whereas nearly all the ergotoxine salts hitherto examined have been obtained crystalline. The salts of both alkaloids form colloidal solutions in water, and are precipitated by electrolytes, so that they are little soluble in the presence of the stronger mineral acids.

### *Physiological*

One of us recently (43) described certain physiological effects—best observed in a cat with the brain destroyed and artificial respiration—which were characteristically produced by a large number of ergot preparations. An analysis of these effects showed that they could be divided into—

(1) A primary stimulation of plain muscular tissues, especially the arteries, the uterus, and the sphincter of the pupil.

(2) A secondary specific paralysis of the motor elements in the so-called 'myoneural junctions' associated with innervation by the true sympathetic system and stimulated by the suprarenal active principle; the inhibitor elements of the same retaining their normal function, as do also the autonomic nerves of cranial and sacral root origin.

The vaso-motor effects may be taken as a typical and easily observed example of this double action. When a powerful dose of one of these ergot preparations (chrysotoxin, commercial ergotinine, etc.) is injected intravenously into a pithed cat the first result is a marked and very prolonged rise of blood-pressure. If, while this rise persists, the sympathetic nerve supply to the arteries is excited at any level, as by faradising the spinal cord or the splanchnic nerves, or injecting intravenously nicotine or a suprarenal preparation, the effect is a very marked *fall* of blood-pressure in place of the customary rise. This particular instance of the action is easy to observe, and is capable

of quantitative application, and we shall frequently refer to the dose of a given preparation causing 'vaso-motor reversal,' meaning thereby the quantity just sufficing to replace the normal pressor effect of a given dose (0.1 mgm.) of the suprarenal active principle by a depressor effect. The accuracy of the measurement is admittedly not great, but it has, at least, a fairly definite end-point, and we have found it preferable to observation of such uncertain effects as those on the cock's comb. At the outset it was necessary to recognise the possibility that more than one active principle might be concerned in the various actions, and for a long time we were engaged in the search for a principle of which we could only postulate that it caused the vaso-motor reversal. Only later were we able to transfer conclusions based on this reaction to the other physiological effects, which the principle, when isolated, was found to produce. In the former communication it was suggested as probable that the primary stimulant action on plain muscle was due to a different principle from that responsible for the secondary sympathetic motor paralysis, although the two were found in close association. This suggestion has recently been supported by Cushny (50), who observed, as we did, that certain pharmacopoeial preparations, such as the liquid extract, produced stimulant effects on plain muscle, resembling, superficially at least, those which we had described, but followed by a disproportionately weak sympathetic motor paralysis. Like ourselves, he regarded this as indicating that the principle responsible for the paralytic effects probably had none of the stimulant properties, and that, where the two sets of effects were observed, two principles were at work. The isolation of ergotoxine, in the form of pure crystalline salts, at once showed, however, that this conclusion was wrong. What, if any, is the relation to ergotoxine of the substance which gives to the liquid extract what specific activity it possesses is at present quite uncertain, and will probably remain so until the substance can be obtained free from other physiologically active principles, such as choline. Possibly the further experiments on ergotoxine, with which we are now engaged, will throw light on the question. However that may be, it is certain that pure salts of ergotoxine produce, in very small dose,



all the effects described in the former paper (43) as characteristic of chrysotoxin, etc.

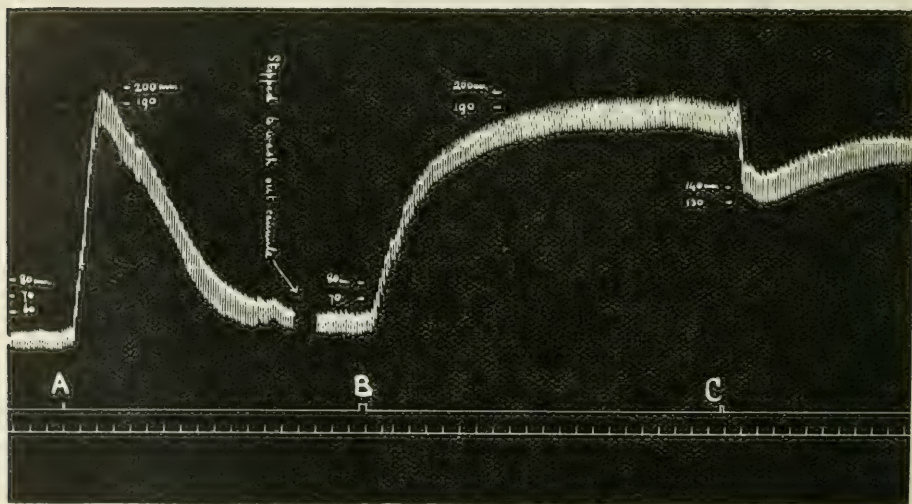


FIG. 1

Pithed Cat, 2 kilos. Artificial respiration. Carotid blood-pressure.

At A—Intravenous injection of 0.5 mgm. of the suprarenal principle.

At B—Intravenous injection of 1 mgm. of ergotoxine phosphate.

At C—Intravenous injection of 0.05 mgm. of the suprarenal principle.

Time marker in this and all the other tracings showed ten seconds intervals.

Reference to that paper will show that, at the time of its publication, we had already found that certain alkaloidal preparations produced the effects in smaller dosage than those of an acidic, resinous nature. The only step needed was the isolation of the alkaloid, in chemically pure form, which was made possible by the discovery that it gave well-crystalline salts. Without repeating the details of the various manifestations of the physiological action already described, it will be sufficient to indicate the relative activity of the pure alkaloid as compared with the impure preparations previously used. As with these impure preparations it was found that, owing probably to the depressant action of the alkaloid on the medullary centres, the stimulant effects were observed in most characteristic form in an



animal with destroyed brain, and under artificial respiration. In a cat under these conditions, 0.5 mgm. of a pure ergotoxine salt per kilo caused the characteristic marked rise of blood-pressure, succeeded by 'vaso-motor reversal.' Some variation occurred with different animals, but the dose necessary to reverse the vaso-motor effect of 0.1 mgm. of the suprarenal principle in the cat did not, in any case, differ widely from this. Rather more—about 1 mgm. per kilo.—was needed to reverse the motor effect of suprarenal or other sympathetic stimulation on the uterus of the cat in early pregnancy: in a case of later pregnancy the dose needed was larger. As with the impure preparations, the sympathetic effects on the heart and the dilator of the iris were comparatively resistant to the paralysis. No quantitative determinations were made with other animals, but, in addition to the species previously examined with the impure preparations (cat, dog, rabbit, ferret, monkey and fowl), observations were made on the pig and the goat.

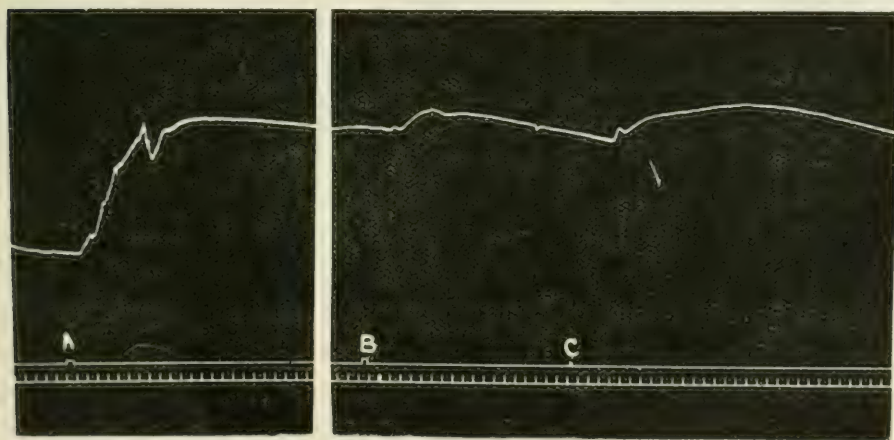


FIG. 2

Cock, 2 kilos. Brain pithed. Artificial respiration. Carotid blood-pressure. Injections intravenous.

At A—5 mgms. of ergotoxine in dilute caustic soda.

At B—5 mgms. of ergotoxine in dilute caustic soda.

At C—0.05 mgms. of the suprarenal active principle.

Note that even 10 mgms. have not obliterated or reversed the suprarenal effect.

In a small pig of 9.5 kilos., with brain destroyed and artificial respiration, intravenous injection of 4 mgms. of ergotoxine phosphate, dissolved in water, caused a rise of blood-pressure from 90 mm. to 140 mm. of mercury ; a further dose of 4 mgms. produced no further pressor effect, and a subsequent injection of 0.1 mgm. of the suprarenal principle caused no longer any rise of blood-pressure, though, on the other hand, no perceptible depressor effect was produced. It appears probable, then, that the vaso-constrictor mechanism in the pig is nearly as sensitive as that of the cat to ergotoxine-paralysis, but that the vaso-dilator element in the sympathetic is insignificant or absent. The pig's bladder gave after, as before the administration of ergotoxine, an inhibitor response to suprarenal and to stimulation of the hypogastric nerves. In the goat the experiment was made under anaesthetic (A.C.E. mixture), with the brain intact. A less marked rise of blood-pressure was produced by injecting ergotoxine phosphate, but the abolition of pressor effect of the suprarenal principle followed, as in the pig, and, moreover, its normal motor effect on the goat's bladder was replaced, after ergotoxine, by distinct inhibition. As pointed out in the former paper, the vaso-motor supply of the cock is particularly resistant to the ergotoxine paralysis. (See Fig. 2.)

Besides the question of the identity of the principles producing the 'stimulant' and 'paralytic' effects respectively, another point was left in doubt in the former paper, namely, the point of action of the stimulus producing the arterial constriction. It was stated there that the effect was produced somewhere peripheral to the spinal cord, and that the slight effect of chrysotoxin, etc., after large doses of nicotine suggested an action on the ganglion cells of the sympathetic system, while, on the other hand, the persistence of the pupillary constriction after atropine indicated, in the case of the *sphincter iridis*, a quite peripheral action. A repetition of the nicotine experiment with pure ergotoxine salts has shown that the pressor effect is quite well produced in the cat after doses of nicotine (30 mgms.) sufficient to abolish the ordinary effects of stimulating sympathetic nerves (such as the pupillo-dilator action of the cervical sympathetic), provided only that the action of the heart has not been too greatly enfeebled by the nicotine injections. (See Fig. 3.)

This indication that the stimulant action on the arteries was also peripheral in origin was confirmed by a plethysmographic experiment. In a cat, with pithed brain and artificial respiration, the right stellate ganglion was excised extrapleurally by Anderson's method.<sup>1</sup> The cat was then turned over, the right fore limb enclosed in a glass plethysmograph, connected with a small Hürthle piston-recorder,

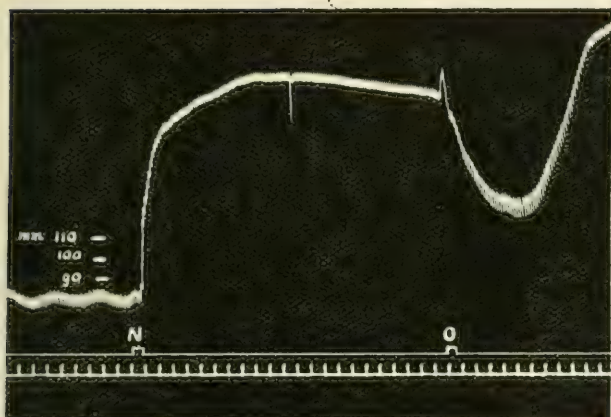


FIG. 3

Cat, 3 kilos. Pithed. Artificial respiration. Thirty mgms. of nicotine had been given. Injections into external jugular.  
At N—1 mgm. of ergotoxine phosphate in water.  
At O—0.1 mgm. of the suprarenal active principle.

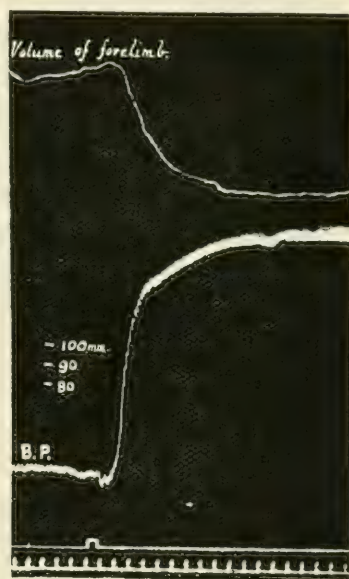


FIG. 4

Cat, 3 kilos. Pithed. Curare. Artificial respiration. Right stellate ganglion excised. Right fore limb in plethysmograph. Carotid blood-pressure.  
At point signalled, 2 mgms. of ergotoxine phosphate, in water, was injected into the jugular vein.

and the blood-pressure recorded from the carotid artery. Two mgms. of ergotoxine phosphate, dissolved in water, were then injected into the external jugular vein. The result is shown in Fig. 4. It will be

1. *Journ. of Physiol.*, XXXI, p. 21 (Proc. Phys. Soc.), 1904.



seen that the rise of general blood-pressure is accompanied by a simultaneous decrease in the volume of the fore limb, which can only have been caused by active constriction of the arteries. As the stellate ganglion had been completely extirpated, the action of the ergotoxine in producing the arterial constriction must have been peripheral on the muscular walls of the arteries themselves. This is in accordance with the result obtained by Jacoby (37) in a perfusion experiment with his preparations. On the other hand, Kobert (25) observed no indication of any effect on the vessels of an excised kidney perfused with a solution of ergotinine in blood, and Dixon (42) found some dilation instead of constriction of peripheral vessels perfused with a liquid extract of ergot diluted with Ringer's solution. It will be clear from what follows that the significance of Kobert's experiment depends largely on the purity of the ergotinine used, and it has already been pointed out that experience obtained with a liquid extract cannot properly be applied to ergotoxine.

There remains the question as to which structure in the arterial wall is the seat of the action. It was pointed out in the former paper that a large dose of an active preparation reverses or obliterates the effect not only of a subsequent suprarenal or nicotine injection, but of a further injection of the ergot preparation itself, whereas barium chloride and pituitary extract still produce their normal actions. This suggests that the stimulant action of ergotoxine is on those elements, the sympathetic motor myoneural junctions, which it subsequently paralyses. Such a conclusion would, of course, be expected on general grounds, and it is supported by the consideration that the stimulant effects are most conspicuous in the case of plain muscular organs which are known to receive motor fibres from the true sympathetic system, viz., the arteries and the uterus. The absence of the pressor effect of ergotoxine after large doses of apocodeine (250 mgms. for a cat) points in the same direction, though the significance of the observation is weakened by the fact that we have never, with the specimens of apocodeine at our disposal, succeeded in obliterating the suprarenal rise of blood-pressure without, at the same time, markedly weakening the action of the heart. On the



other hand, ergotoxine very distinctly stimulates plain-muscular organs in which there is no reason to suspect motor innervation from the true sympathetic. Thus it causes a very marked constriction of the cat's pupil, and a variable contraction of the urinary bladder in the same animal. In the case of the sphincter of the iris the motor nerve-supply from the cranial nerve can be excluded completely by atropine, and there is no reasonable escape from the conclusion that the ergotoxine here acts directly on the muscle-fibres, and the same very probably holds good for the less marked effects on the urinary bladder and the stomach and intestines. But, whatever may be the exact point of action of ergotoxine, there is no good reason for regarding any of its effects on plain-muscular organs as other than peripheral in origin. The effect on the heart itself appears to be a slight one. In a few experiments made with the isolated hearts of rabbits and cats, perfused with Locke-Ringer solution, a change to the same fluid containing 1 in 50,000 ergotoxine hydrochloride caused, in some cases, a small increase in the force of the beat; in some cases no obvious effect. In the anaesthetised animal, with medullary centres and vagi intact, ergotoxine causes an obvious vagus-inhibition of the heart, similar to that which Kobert observed with cornutine. Even with the medulla destroyed, or the vagi cut, the sudden and prolonged rise of blood-pressure, unaccompanied by any marked action on the accelerator mechanism, often produces a marked irregularity of the beat, unless large doses of atropine have been given.

Apart from these effects on decerebrate or anaesthetised animals, the general toxic effects have been studied by injection into frogs, fowls, rabbits, and cats. The action on frogs is of importance in considering the relation of ergotoxine to other ergot alkaloids. According to Kobert, cornutine has a very characteristic action on frogs, producing in doses of as little as  $\frac{1}{32}$  mgm. strychnine-like spasms, succeeded by a veratrine-like paralysis. The effects of ergotoxine are very slight as compared with those attributed to cornutine. One to two milligrammes of ergotoxine phosphate dissolved in distilled water, or of the free base dissolved in dilute sodium hydrate, caused an initial trace of increased excitability, succeeded, in the course of a

few minutes, by a diminution in the power of flexing the hind limbs. The following record shows the sequence of events :—

FROG, 29 grammes.

- 12.40 p.m. 1 mgm. of pure ergotoxine phosphate, dissolved in 1 c.c. distilled water, injected into dorsal lymph sac.
- 12.41 p.m. Frog executes one or two leaps, which appear unusually high, and croaks while jumping.
- 12.42 p.m. Flexion of hind limbs performed slowly. Frog, in alighting after a leap, fails to support itself on its fore legs and falls flat on the table. Respiration rather feeble.
- 12.43 p.m. Jumps feebly, hind limbs being obviously trailed after the jump, and only slowly drawn up again.
- 12.44 p.m. Progresses by crawling in preference to jumping. Placed on back does not attempt to turn over. Respiration slow but vigorous.
- 12.52 p.m. Turns over when placed on back. Respiration more rapid. Flexion of hind limbs still markedly defective. Killed by pithing.

After doses up to 5 mgms. the frog gradually recovered during the succeeding two or three days. No attempt was made to determine the lethal dose. A prominent feature in the effect was the ease with which the animal became fatigued. After 2 to 3 mgms. the frog could for some time execute a fairly powerful leap, with prolonged extension of the hind limbs. A few such leaps, however, performed at intervals of a few seconds in response to irritation of the circumanal skin, exhausted the animal, so that no further leaps could be executed until after a rest of several minutes. That this fatigue was in part peripheral was shown by the records obtained with muscle-nerve preparations from such frogs. Even the first twitch-curve recorded after preparation showed considerable prolongation, especially of the last part of the relaxation. These peculiarities were accentuated by giving a series of stimuli at intervals of a few seconds. The great prolongation of the last part of the relaxation is shown very markedly in a fatigue-record taken with a slowly moving drum and stimuli at intervals of two seconds. The second stimulus occurs long before the lever has returned to the base line, so that the first five or six twitches are performed with increasing internal support. The muscle behaves, in fact, very similarly to one already exposed to fatigue.

Since this effect had some features in common with the veratrine-like action attributed to cornutine by Kobert, who found that, with large doses, the preliminary excitant effect might be overshadowed or lost altogether, an attempt was made to produce the true cornutine convulsions by giving very small doses of ergotoxine;  $\frac{1}{10}$ ,  $\frac{1}{20}$ , and  $\frac{1}{30}$  mgm. of ergotoxine phosphate, injected into frogs of 17, 12, and 30 grammes respectively, produced only the same symptoms as the larger doses in a weak and evanescent form. There seems no room for doubt, therefore, that, while the cornutine of Kobert may have contained, indeed, almost certainly did contain, some ergotoxine, the most characteristic of its effects on the frog must have been due to some other constituent.

The effects of ergotoxine on mammals, when injected intravenously or intramuscularly, presented a rather greater resemblance to the cornutine effects described by Kobert. In the rabbit the effects varied considerably in intensity in different individuals, and it was not possible to assign any accurate value to the lethal dose. The following experimental records indicate the general course of the action:—

(1) RABBIT, 1790 grammes.

- 12.10 p.m. 3 mgms. of ergotoxine phosphate in 1.5 c.c. of distilled water injected into lateral vein of the right ear.
- 12.11 p.m. Ears very pale. Jerky movements of the legs.
- 12.15 p.m. Ears no longer pale. Restless, jerky movements continue.
- 12.18 p.m. Marked tendency to sprawl on the table with legs outspread. Head depressed. Feet seem to slide away from the animal till it sprawls flat, when there is a sudden, jerky recovery of the more normal crouching attitude. This is repeated frequently.
- 12.31 p.m. Twitching of ears and eyelids, teeth chatter.
- 12.35 p.m. Much mucous secretion in nose, and saliva in mouth. Noisy breathing.
- 12.37 p.m. Legs completely extended on table: occasional convulsive movements. Rabbit has frequently, since the injection, passed moist faecal pellets.
- 12.46 p.m. Animal feels hot. Temperature taken in rectum and found to be  $44^{\circ}$  C.
- 12.49 p.m. Respiration ceases.
- 12.50 p.m. Thorax opened. Heart still faintly beating. Right ventricle greatly distended. No clots in heart or great vessels.

The high temperature at and after death seemed to be a characteristic effect of fatal doses. In another rabbit of 2950 grammes, an intravenous injection of 10 mgms. of the



pure alkaloid caused death in 1 hour 25 minutes with post-mortem temperature of  $44.5^{\circ}\text{C}$ . It is of interest to mention, in this connexion, that Zutz (36), with a commercial specimen of 'Cornutin citrate,' observed no rise of temperature in a guinea-pig, which, after an injection of 10 mgms., recovered in  $1\frac{1}{2}$  hours.

(2) RABBIT, 1410 grammes.

12.4 p.m. 1 mgm. of ergotoxine phosphate dissolved in 0.5 c.c. of distilled water injected into right ear-vein.

12.12 p.m. No symptoms except very rapid respiration and prominence of the eye-balls.

1.10 p.m. Respiration still rapid. Jerky movements begin.

2.0 p.m. Recovered.

(3). RABBIT, 1420 grammes.

11.55 a.m. 2 mgms. ergotoxine, dissolved in 1 c.c. dilute NaOH, injected into right ear-vein, 2 mgms. similarly into left ear-vein. Altogether, therefore, 4 mgms.

11.58 a.m. Very rapid respiration and twitching of the limbs.

12.5 p.m. Fore-legs unable to support the animal, which sprawls with its nose on the ground. Persistent fine tremors and occasional twitching of the limbs.

12.42 p.m. All legs extended. Dyspnoea continues.

12.56 p.m. Rectal temperature,  $42^{\circ}\text{C}$ .

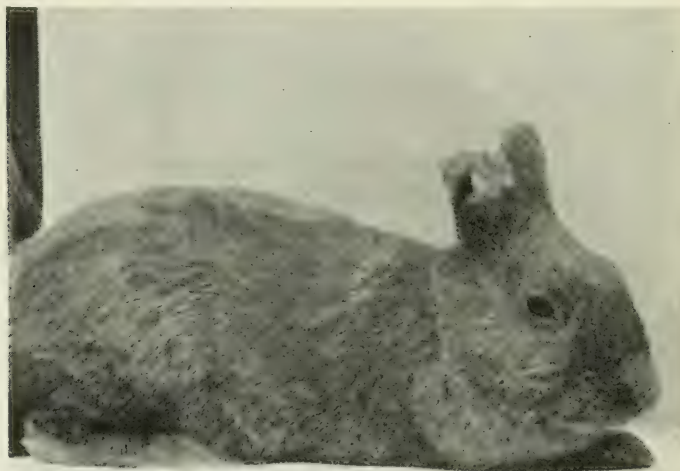


FIG. 5

Rabbit, showing ears shortened by gangrene two months after injection of 4 mgms. of ergotoxine.



From this time, until recovery begins, animal lies with fore limbs spread wide and hind limbs extended. The latter are occasionally and alternately flexed and again extended, producing a wobbling of the hinder part of the animal on the fore part, which remains stationary, supported on the broad base formed by the chest and the spread fore limbs. The head is at first depressed, with nose on the table: later (2.30 p.m.) it is raised. At 3.30 p.m. profuse salivation was noticed, saliva dropping from the mouth on to the floor. Respiratory sounds indicated bronchial secretion. About 5 p.m. recovery began. Animal could now draw up the hind legs and the dyspnoea was less severe. Next morning recovery was practically complete, and the animal remained apparently normal for a fortnight. It was then noticed that the skin of the peripheral two-thirds of each ear was becoming darker in colour, greasy in appearance, and losing its hair. A week later a distinct, sinuous line of demarcation separated the healthy proximal from the now obviously gangrenous peripheral portions. These peripheral portions gradually dried and were ultimately shed, without any bleeding, a month after the first appearance of the gangrene, and therefore six weeks after the single injection. A photograph, reproduced in Fig. 5, was taken, and the rabbit then killed and the head preserved.

This occurrence of true gangrene in the rabbit, after administration of an ergot-preparation, appears to be unique: we have, at any rate, been unable to find another case in the literature, though Kobert mentions the occurrence of subcutaneous bleeding in a rabbit's ear as a result of sphacelinic acid. Nor have any of the other rabbits which received sub-fatal doses of ergotoxine in our experiments shown any trace of such an effect. In one case an attempt was made to produce the gangrene by repeated sub-fatal doses as follows:—

(4) RABBIT, 1550 grammes.

January 9th. 2 mgms. of pure ergotoxine phosphate in 1 c.c. of distilled water, injected into the right ear-vein. The animal showed the usual dyspnoea and restless, jerky movements. On the morning of January 10th it had quite recovered and was given a further injection of 2 mgms. intraperitoneally. The usual symptoms followed in a milder form. The animal was then left till February 15th. By this date the weight had risen to 1900 grammes and the animal seemed quite well and showed no trace of gangrene.

February 15th. Weight 1900 grammes. Temperature 38.7 Seems perfectly normal. Heart-beat 300 per minute.

11.40 a.m. 2 mgms. ergotoxine phosphate in 1 c.c. distilled water injected into left ear-vein.

11.41 a.m. Heart-beat 280.

11.42 a.m. Animal very restless, with jerky movements.

11.47 a.m. 2 mgms. ergotoxine phosphate in 1 c.c. water injected into right ear-vein. Injection immediately followed by curious movements, the hinder part of the animal raised on the rather rigid hind limbs, while the fore feet are flexed so that the animal is supported on the wrists.

- 11.51 a.m. Heart-beat 160 per minute. Animal now holds itself high off the table, the body being moved restlessly from side to side and the muzzle depressed between the fore limbs.
- 12.0 noon. Restless movements continue: the characteristic sliding sprawl of the fore limbs, with jerky recovery, has appeared. The advanced position of the hind feet leads to a gradual movement backwards to the edge of the table.
- 1.30 p.m. The restless movements have almost vanished. The animal appears to be hyper-excitable, a slight touch on the back eliciting a sharp contraction of all the muscles of the body.
- 2.10 p.m. 20 c.c. dark coloured turbid urine passed. No albumen.
- 2.45 p.m. Less excitable. Fore legs still sprawl, so that chest lies on the table. Head raised. Respiration very rapid (340 per minute), and so vigorous as to shake the whole animal. Temperature 40.9° C.
- 2.50 p.m. Marked weakness, animal lying with all limbs extended. Moist faecal pellets passed. Anal sphincter apparently quite competent.
- February 16th. Animal seems rather weak, but otherwise normal. Faeces still moist.
- 12.19 p.m. Heart beat 280 per minute. Respiration 100 per minute. 2 mgms. of ergotoxine phosphate in 1 c.c. distilled water injected into left ear-vein. Heart-beat irregular. Characteristic position and movements.
- 12.27 p.m. Injection of 2 mgms. similarly into right ear-vein. Rigidity of hind limbs and jerky movements intensified. Heart-beat 100 per minute. Respiration very rapid and shallow with marked nasal movements: could not be accurately counted.
- 2.45 p.m. Only slight hyper-excitability remained.

Several further injections were given, with diminishing severity of result. The following were the doses and other details:—

February 18th. 4 mgms. Salivation noticed for the first time with this rabbit.

February 19th. 5 mgms.

February 20th. 5.5 mgms.

February 21st. 5 mgms.

February 22nd. 10 mgms. The resultant nervous symptoms were not more severe than those originally produced by 4 mgms. On February 23rd the weight, which had been practically constant at 1900 grammes since February 15th, had risen to 1920 grammes, and by February 25th again to 2000 grammes.

February 25th. 15 mgms. in all injected, 10 mgms. into the ear-veins, 5 mgms. hypodermically. The effect was again rather less marked than that caused by 10 mgms. on February 22nd. With each injection subsequent to that on February 15th the animal passed normal urine a few minutes after the injection. This animal is still (April 2nd) under observation, and up to the present has shown no signs of the development of gangrene or any other bad effect as the result of the repeated injections, amounting in all to 56.5 mgms. of the pure phosphate.

There is clear evidence in this last case of the development of tolerance with repeated administration of the alkaloid. As already indicated, the reaction of different individuals showed variation, but intravenous injections of 5 mgms. per kilo. body-weight, such as were ultimately borne by this rabbit with only temporary symptoms of intoxication, have, in our experience, always proved fatal within two hours, if given as an initial dose.

The symptoms exhibited by the cat, when ergotoxine was given intramuscularly to the intact animal, were, as might have been expected, more striking and characteristic than those seen in the rabbit. The following are records of the three experiments made:—

(1) MALE CAT, 3450 grammes.

12.26 p.m. 5 mgms. of ergotoxine phosphate in distilled water injected into the thigh muscles.

12.32 p.m. Cat seems unwell. Mews repeatedly.

12.33 p.m. Vomits.

12.35 p.m. Obviously ataxic and uncertain in its gait.

12.38 p.m. Unable to stand: falls if placed on its feet.

12.44 p.m. Lies on one side with the head raised from the floor. Occasionally moves the head and fore limbs. If placed on its feet at once rolls over.

12.56 p.m. Lies quiet if not touched, but, if touched, is evidently hyper-excitable, starting violently.

1.4 p.m. Marked salivation.

1.15 p.m. Becoming drowsy.

1.16 p.m. Salivation very profuse, watery saliva dropping from the mouth. Pupils becoming constricted.

2.0 p.m. Respiration 240 per minute. Mouth open; salivation continues.

2.30 p.m. Pupils are now intensely constricted, and no longer dilate at all when the eyes are shaded.

2.50 p.m. Rectal temperature 42° C.

4.0 p.m. Lies on its side still, but can just stand unsteadily if placed on its feet. Right pupil still minimal; left dilates slightly in the dark. Thin slime passed continually from the anus. Obvious paralysis of the internal anal sphincter, the lightest pressure on the abdomen causing the passage of slimy semi-fluid faecal matter. Firmer pressure needed to express the urine, the urethral sphincter being more competent.

6.0 p.m. Cat seems better and holds up its head. Pupils now dilate slightly in the dark. Respiration deeper and slower—136 per minute. Heart-beats very powerful—167 per minute. Anal sphincter still incompetent. Rectal temperature 41.5° C.

The cat was found dead at 8.30 next morning. The lungs were found very greatly congested; the abdominal lymphatics were very red, as was also the suprarenal medulla.

(2) LARGE FEMALE CAT IN ADVANCED STAGE (LAST WEEK) OF PREGNANCY.

August 23rd.

2.42 p.m. 3 mgms. of ergotoxine phosphate in distilled water injected into the thigh muscles. Cat rapidly became sleepy and lethargic, but otherwise showed nothing abnormal at first.

3.11 p.m. Passed a large volume of urine.

3.18 p.m. The large horns of the uterus, which had previously felt soft, could be felt, through the abdominal wall, to be hardening round the embryos.

3.30 p.m. Pupils are now contracted. Cat lying down and very sleepy. Uterus feels very hard and tightly contracted round the foetal sacs, which have been pushed tailwards by the contraction.

3.45 p.m. Uterus still tightly contracted. Cat rises to its feet, but soon sinks back on to the floor.

5.0 p.m. Condition the same, except that the pupils now dilate slightly when shaded.

August 24th.

8.0 a.m. The cat had already borne two dead, almost fully developed kittens; at 8.30 a.m. a third was born without difficulty, and found to be also dead. The placenta was in all cases expelled normally and without haemorrhage. Cat very sleepy all day.

August 25th. Cat quite well and moving about normally.

(3) FEMALE CAT, 3420 GRAMMES, ABOUT THE MIDDLE OF PREGNANCY.

August 24th.

10.30 a.m. 3 mgms. of ergotoxine phosphate in distilled water injected into the muscle of the thigh.

10.35 a.m. Respiration shallow and irregular. Commencing ataxia.

10.45 a.m. Respiration more rapid. Cat is markedly ataxic, and also hyper-excitable, jumping and spitting if touched lightly.

10.50 a.m. Profuse salivation, saliva dropping from the mouth.

11.0 a.m. Sphincter and internus paralysed, slimy faeces being expressed by slight pressure on the abdomen.

2.0 p.m. Uterus obviously contracted. Sphincter of pupil constricted.

5.0 p.m. Uterus now feels hard and tightly constricted round the foetuses. The other symptoms passing off.

August 25th.

9.30 a.m. Blood-stained watery fluid has been passed from vaginal orifice, and is still being passed slowly. No foetuses in the cage. Abdominal palpation reveals no uterine horn on the right side, where formerly it was felt with two foetuses. On left side a large, soft, elongated mass can be felt. Cat seems fairly well.



August 27th. Condition still the same. Blood-stained fluid still trickling from the vagina. Cat seemed unwell, and was killed by chloroform.

On post-mortem examination all organs seemed normal except the uterus. Of this both horns were found on the left side of the animal. They were somewhat soft and 'boggy,' and blood-stained. The right horn contained two foetuses in a common bag of membranes. The amniotic fluid was blood-stained, and the foetuses, dark red and very soft, had obviously been dead for some time. The placentae were easily separable, soft and friable, and chocolate-coloured. The vagina was normal.

The fact being thus established that ergotoxine causes, in the intact pregnant cat, a tonic contraction of the uterus, we did not consider it worth while to pursue this particular line of experiment further. Several observers have laid much stress on the regular production of abortion as an essential characteristic of the active therapeutic principle of ergot. The claim of the ergot alkaloids to be regarded as such therapeutic principles has recently been adversely criticised by Kraft from this point of view. But, as has already been pointed out elsewhere—Barger and Dale (47)—the experiments recorded in his paper were made on rodents, which are relatively very insensitive to the action of ergotoxine. Moreover, in any case, there seems little warrant in the historical accounts of epidemics of ergotism, in medico-legal records, or in the practical use of ergot in obstetrics, for expecting of the active principle that it shall, in all cases, and at any period of pregnancy, produce abortion. In the four cases described by Kobert (20) of pregnant women who had taken ergot with the object of inducing abortion, death in all cases supervened before abortion had occurred, and in only one case had the expulsion of the foetus even begun.

Historical evidence seems to us to be in no way opposed to the view that abortion, following ergot-ingestion, is due to the production of a tonic uterine contraction, leading to asphyxiation of the embryo, followed, in the natural course, by its expulsion. We consider it sufficient, therefore, to have shown that large doses of ergotoxine can produce contraction of the pregnant uterus, at a time when such contraction is opposed to the natural, physiological tendency of the organ. That the foetuses, killed by the uterine tonus, may be, in some cases, retained days after the immediate effect of the drug has

passed off, seems to us to be of little consequence. The important conclusion, from the practical point of view, is that the alkaloid, in doses too small to cause general symptoms, might be expected to reinforce the natural tendency of the uterus to tonic contraction *post partum*. Whether that conclusion is justified must be determined by clinical experience.

The domestic fowl has been more frequently, perhaps, the subject of experiment in investigations on ergot than any other animal. It was of particular interest and importance, therefore, to determine the action of ergotoxine on the cock.

Kobert, finding that cornutine, like sphacelinic acid, caused a rise of blood-pressure, was surprised at the failure of the alkaloid to cause gangrene. On the other hand, he found that 4 mgms. of the alkaloid, given hypodermically to a cock of  $2\frac{1}{2}$  kilos., caused narcosis, and, about two and a half hours after the injection, death in convulsions.

This marked convulsant effect on the fowl, like the similar effect on the frog, was not, in our experience, obtained with ergotoxine. The effect of this alkaloid, indeed, showed considerable variations with different individuals. Although our attention was not deliberately directed to the point, and though no attempt was made to use thoroughbred specimens, we got the impression that there are considerable differences in the reaction of different breeds of fowls. The following are typical examples of a number of experimental records<sup>1</sup> :—

(1) BLACK COCKEREL, with bright red comb.

July 18th.

12.26 p.m. 20 mgms. of ergotoxine phosphate in 5 c.c. distilled water injected into the breast muscle.

12.30 p.m. Comb becoming paler, especially at the root. Skin round the eyes quite pale.

12.31 p.m. Beak open : breathing quick and laboured.

12.45 p.m. Comb still pale over the eyes : tips rather darker, and bluish in tinge. Wattles pale and cold. Condition continued much the same during the afternoon.

1. The injections were kindly performed by Mr. C. T. Symons.

- 5.15 p.m. Skin round the eyes and the root of the comb are still pale. Elsewhere the comb is of a dusky purple colour, mottled with redder patches. The bird feels hot. Temperature under the leg  $43^{\circ}\text{C}$ .; in the cloaca  $44.5^{\circ}\text{C}$ .

July 19th.

- 10.0 a.m. Comb red, except for a rather sharply-defined, blackish-purple area at the hinder margin, and the tips of the two last digitations, which are also blackish. The cock seems fairly well and walks normally.
- 5.36 p.m. 10 mgms. of ergotoxine in 2.5 c.c. of dilute NaOH injected into the wing-vein.
- 6.0 p.m. Symptoms similar to those after the injection on the previous day.

July 20th.

- 11.0 a.m. The whole of the comb bluish-red in colour, darker at the tips of the digitations. Just over the eyes a portion is of the normal red colour. The cock breathes slowly with closed beak, and seems very drowsy, the eyes being closed except when the bird is roused. It squats on the ground, and, if dropped one foot to the floor, stumbles in alighting.
- 2.45 p.m. Just living but collapsed. Respiration slow and regular. Comb darker purple and cold.
- 2.50 p.m. A few convulsions, leading to death.

*Post-mortem*.—The skin over the breast was found discoloured, being green and dark-red in patches. The peritoneal cavity contained a green offensive-smelling fluid. The whole of the small intestine was congested, and the mucous membrane reddened, in places haemorrhagic and ulcerated. At the tip of the duodenal loop was an oval perforation, about 1 cm. in length, obviously the result of ulceration. The comb was dusky purple in colour, but had not in any part become dry and shrivelled.

- (2) SMALL BLACK COCK (Cross-bred Minorca), with thin, erect comb; weight 1700 grammes.

July 18th.

- 3.5 p.m. 40 mgms. of ergotoxine phosphate, in a gelatin capsule, given by the mouth.
- 4.0 p.m. Hinder part of the comb seems slightly darker, the rest slightly paler than before. The effect is not at all pronounced, and is accompanied by no other abnormal symptoms.
- 5.45 p.m. Condition unchanged.

July 19th.

- 9.30 a.m. The cock is normal. The hinder end of the comb appears a trifle bluish, but not more so than it often appears in normal birds.
- 3.3 p.m. 7 mgms. of ergotoxine phosphate, dissolved in distilled water, injected into the wing-veins.



3.5 p.m. The root of the comb pale, as is also the skin round the eyes. Head depressed. Wings drooping. Beak open. Some salivation. Slight dyspnoea. This condition continued for the rest of the afternoon.

July 20th.

11.0 a.m. The tips and hinder part of the comb are quite dark, especially the small papillae. The flat expansion behind the last digitation is bluish in colour. The rest is normally red, but feels cold. The lower part of the wattles is dusky and cold.

5.30 p.m. Cock rather sleepy. Comb unchanged.

July 21st. The whole comb red again except hinder edge and tips of last two digitations. Cock seems well and vigorous.

11.7 a.m. 10 mgms. of ergotoxine phosphate in distilled water injected, 5 mgms. hypodermically into the breast, 5 mgms. intravenously into the jugular vein.

Effects as before appearing a few minutes after injection, whole comb becoming dusky purple, except a pale area at the root continuous with the pale skin round the eyes. Constitutional symptoms similar to those following previous injection.

4.0 p.m. Ataxia, dyspnoea, and prostration have disappeared, the cock strutting about normally. Comb red, except the hinder part, which is still purple, the tips of the two last digitations being black.

July 23rd.

10.0 a.m. Whole comb bright red and warm, except a narrow band round hindmost flat expansion and the tips of the two hindmost triangular digitations. These parts are all black, dry, slightly shrivelled, and quite insensitive. The lower edge of the left wattle shows the same condition.

12.5 p.m. 10 mgms. of ergotoxine in 4 c.c. dilute NaOH injected into the breast muscle. The comb showed the usual changes. The constitutional symptoms were less marked than with former injections, salivation being the most prominent.

July 24th. Cock seems fairly well. Weight has fallen only to 1620 grammes. The black area at the hinder end of the comb is more extensive. 10 mgms. of ergotoxine in dilute NaOH again injected into the breast muscle, the usual changes in the comb following, but the constitutional effects being again less severe.

July 25th. Still further extension of gangrene. Otherwise the bird seems normal. 10 mgms. of ergotoxine phosphate in distilled water injected under the skin of the neck. The usual changes in the comb appeared, developing rather slowly. Constitutional effects slight.

July 26th. Comb still purple, except at the root, where it is pale. Intense pallor of the skin round the eyes. The gangrenous process is extending. The wattles are pale at the root. Below they are swollen and purple, and the skin is peeling (moist gangrene).



July 27th. Most of the comb again red. The black area at the hinder end is now broad and sharply demarcated. The dry gangrene of the two last digitations is advancing. The lower ends of both wattles show the moist gangrenous condition, and, continuous with this, a broad flat tip of dry gangrene on the left hand side. Cock weighs 1470 grammes.

The bird was kept under observation. The weight fell to 1300 grammes on July 30th, and then increased to 1450 grammes again by August 2nd. On August 9th the condition was unchanged, except that the line of separation of the gangrenous portions was becoming gradually more definite. A further injection of 10 mgms. of ergotoxine was given into the breast muscle. The usual comb-changes were produced in a slighter form, and by August 10th the bird was as before.

On August 11th the gangrenous portion of the lower end of the wattle was found to have fallen off. The line of separation was perfectly healed, and there had been no bleeding. The tips of the digitations and the area at the hinder end of the comb separated on September 1st and 13th respectively. The abbreviation of the comb and wattles remained very evident till the spring, when the loss was rapidly repaired.

The following is an instance of acute intoxication :—

- (3) WHITE COCK, weighing about 2 kilos. Comb erect and scarlet. Skin round the eyes and wattles also bright scarlet.
- 1.11 p.m. 5 mgms. of ergotoxine phosphate in 2.5 c.c. of distilled water injected into the left wing-vein.
- 1.12 p.m. 4 mgms. similarly injected into the right wing-vein. There was immediate ataxia, and simultaneously the skin round the eye and the root of the comb became pale, the rest of the comb darkening.
- 1.14 p.m. Intense ataxia. Head held down on the table with open beak, wings drooped ; bird cannot support itself on its legs.
- 1.19 p.m. Whole comb and wattles very dark purple. The tips of the digitations and the hinder end of the comb are especially dark. Very marked dyspnoea as well as ataxia. The prostration gradually advanced, the breathing becoming more feeble till at 2.1 p.m. it stopped.

*Post-mortem Examination* at 3.0 showed some congestion of the bowels. The right auricle and ventricle, and also the left auricle, contained large clots. The left ventricle was quite empty. The lungs congested, but less so than was expected.

A number of attempts to induce gangrene failed, owing to the death of the bird when the effect was just beginning. Although the dose given by the mouth to the cock in Experiment (2) had been without obvious effect, it was thought that it might be connected with the ultimate successful production of gangrene in that experiment. Another cock was, therefore, given large doses (in all 80 mgms.) of ergotoxine by the mouth, and, when this had proved without effect, subsequent

intravenous and intramuscular injections. The usual temporary discoloration of the comb, attended by ataxia and dyspnoea, were produced, but no true gangrene followed.

The following experiment, undertaken with the idea of producing the gangrene by repeated small doses, is chiefly of interest as indicating the severe effects of even small doses of the pure alkaloid :—

- (4) COCK, WITH SPECKLED PLUMAGE, very large erect red comb and large pendulous wattles. Weight 1770 grammes. 10 mgms. of the phosphate of ergotoxine, purified by repeated recrystallisation, were dissolved in 0.5 c.c. of absolute alcohol, four drops of 10 per cent. NaOH added, and the solution then made up with water to 5 c.c.
- 3.1 p.m. 1 c.c. of this solution, containing ergotoxine corresponding to 2 mgms. of the phosphate, injected into the left wing-vein. The skin round the eye immediately became pale.
- 3.2 p.m. Wattles also very pale. Bird crows loudly and walks about the room without ataxia.
- 3.4 p.m. Wattles livid. Pallor spreading to the root of the comb.
- 3.5 p.m. Gait becoming ataxic.
- 3.10 p.m. Ataxia very marked. Head drooped forward, beak open, and salivation profuse. Whole comb, with the exception of a patch at the front end, dusky purple in colour.
- 3.25 p.m. Ataxia has practically disappeared, the bird being now only sleepy. Root of the comb is pale, the rest dusky purple, the darkest portions being, as usual, the hinder flat expansion, the tips of the last three digitations and the lower ends of the wattles.
- 4.40 p.m. The front part of the comb is recovering its normal colour in patches.
- 4.42 p.m. Another 2 mgms. injected into the left wing-vein. The symptoms were reproduced, but the ataxia was more evanescent. Salivation again marked.
- 5.18 p.m. 6 mgms. in 3 c.c. injected into the breast muscle. The ensuing ataxia was less pronounced, but more persistent, being quite perceptible on the following morning, as was also the discoloration of the whole comb, and the excessive salivary secretion.

At 10.24 on the next morning a further injection of 4 mgms., similarly dissolved, was made into the breast-vein. By 12.15 the ataxia resulting had nearly disappeared, and salivation had ceased. The comb presented the dusky colour, the intensity of the change having the usual distribution.

At 12.59 p.m. 3 mgms. of ergotoxine were given intravenously. The ataxia quickly passed off. At 6 p.m. the hinder end of the comb and the digitations were very dark. The bird seemed sleepy, but otherwise not unwell.

On the following day the bird was found dead in its cage at 3 p.m. Post-mortem the only abnormality found was an inflamed condition, acute in patches, of the mucous

membrane of the whole intestinal tract. The proventriculus showed follicular catarrh. The crop was not noticeably affected. The comb was pale, but fairly normal in colour, except for thin black strips at the anterior and posterior ends, and the black tips of the digitations.

It was evident that this administration of 17 mgms., spread over two days, was still too rapid to give time for the development of gangrene before the death of the animal. It was thought that absorption should take place more slowly if the free alkaloid were dissolved in alcohol and given hypodermically. The insoluble base, precipitated as the alcohol became mixed with the tissue fluids, should, it was thought, be slowly absorbed, time being thus given for the development of a more chronic action. That this expectation was justified is shown by the following experiment :—

BLACK COCK, weighing 2120 grammes, with large bright red comb.

100 mgms. of pure ergotoxine oxalate was dissolved in 3 c.c. of absolute alcohol. To this was added an equivalent of  $\text{Na}_2\text{CO}_3$  in distilled water. The precipitate of sodium oxalate was filtered off. The filtrate was made up to 10 c.c. with absolute alcohol, thus giving a solution of the free alkaloid in 80 per cent. alcohol.

September 17th.

3.20 p.m. 1 c.c. of the above alcoholic solution, containing 10 mgms. of free base, injected under the skin of the breast.

3.22 p.m. Skin round the eye becoming slightly pale. The comb became very slowly pale at the root and dusky elsewhere. By 6.0 p.m. the duskiness of the comb was marked, but the bird seemed quite well and vigorous.

September 18th.

11.15 a.m. The whole comb was still rather dark in colour, the tips of all the digitations being black.

11.33 a.m. A further 1 c.c. of the same alcoholic solution of ergotoxine injected hypodermically. The onset of the effect was again gradual. By 5.15 p.m. the whole comb and wattles were blackish purple in colour, except two small red patches in the middle of the comb.

September 19th.

11.30 a.m. The body of the comb is again red, with black patches. The skin round the eyes is still rather pallid. *All the digitations are black*, and their tips are beginning to dry. The bird seems fairly well, and shows no sign of ataxia. Weight 2070 grammes.

September 20th.

10.45 a.m. Weight 2080 grammes. All the comb has regained its natural colour, with the exception of the digitations which are all intensely black, and drying at the tips, as much as 0.5 cm. being dry and shrivelled.

10.55 a.m. Hypodermic injection of a further 10 mgms. of ergotoxine in 80 per cent. alcohol. The effects were again similar, and the gangrenous process was found to have advanced on the 21st, when a further 10 mgms. were injected. After this last injection the hinder part of the comb remained dark in colour till the 25th, when 20 mgms. were injected in similar solution. The whole comb then remained dark purple for forty-eight hours, when the effect again gradually receded, leaving practically the whole of the part of the comb behind the root black and drying. This, with the last three digitations, was shed in one piece about a fortnight later. The tips of the digitations anterior to this were similarly separated.

Fig. 6 shows a photograph of this bird taken in the following March, when the comb was beginning to grow again.



FIG. 6

Cock, with comb reduced by gangrene (see text). The shape of the comb before the experiment is roughly outlined.

Throughout this experiment the bird exhibited no well-developed ataxia, no disorder of the digestive tract, and apart from the effect on the comb, remained practically in normal health.



The resemblance between the effects of this administration of ergotoxine, by a method ensuring slow absorption, to those obtained by Kobert with sphacelinic acid given in initially small but gradually increasing doses, is very striking. The effects in the earlier experiments, on the other hand, when the ergotoxine was injected in water-soluble form, are more reminiscent of those which he obtained with large initial doses of sphacelinic acid.

It follows that Kobert's conclusions as to the part played by sphacelinic acid in the epidemics of ergotism can, for the most part, at any rate, be transferred to ergotoxine. We are not at present in a position to discuss the conditions which determine the absorption of ergotoxine from the alimentary canal when it is mixed with other substances, as in sphacelinic acid or native ergot, whereas an equivalent amount of a pure ergotoxine salt is practically without effect when given by the mouth. It is less difficult to interpret the powerful action of the pure salts of ergotoxine given hypodermically as compared with its comparatively feeble effect when injected in a resinous mixture such as sphacelinic acid (Kobert). There is no room for doubt, however, that under conditions which ensure its satisfactory but not too rapid absorption ergotoxine is the cause of the gangrenous type of ergotism. On the other hand, its relation to the convulsive nervous type is less clear. Nervous symptoms, indeed, are prominent in acute poisoning by ergotoxine injected intravenously; but with slow administration we have never seen anything suggestive of convulsive ergotism, in which it is possible that, as Kobert suggested, a different active principle is concerned. Of particular interest in connection with the action of ergotoxine are certain diseases which have been mentioned as possibly due to ergot poisoning (see Ehlers (34)). One of such is Raynaud's symmetrical gangrene, which, it has been suggested, may be a sporadically occurring delayed ergotism. The case of the one rabbit, in which we observed gangrene some weeks after a single non-fatal injection of ergotoxine, is an interesting parallel.

Still more interesting, in view of our description of sympathetic motor (including vaso-motor) paralysis as the most characteristic

effect of ergotoxine, is the connection, suggested by Ehlers, between ergot and the disease described by Weir Mitchell as erythromelalgia, in which vaso-motor paralysis in the extremities is the most prominent symptom.

## THE RELATION OF ERGOTOXINE TO OTHER ERGOT ALKALOIDS

### (a) *Hydro-Ergotinine*

In a private communication to us, Herr Kraft states his agreement with our conclusion that the alkaloid which he described as hydro-ergotinine is identical with our ergotoxine.

### (b) *Ergotinine*<sup>1</sup>

As Tanret's crystalline ergotinine was the first well-defined alkaloid in ergot, it was, not unnaturally, assumed by its discoverer to be the active principle. Experiments on animals, made in France, to some extent seemed to support this view. Since Tanret, however, applied the name 'ergotinine' also to the amorphous mixture which contains the highly active 'ergotoxine,' it is obvious that no weight can be attached to experiments of which it is not definitely stated that the specimen used was wholly crystalline. On this ground the clinical results of Chahbazian, and the more recent physiological experiments of Plumier, must be set aside. On the other hand, Kobert, who used a pure specimen, consisting of 'very beautiful white needles,' prepared by Tanret himself, found only a trace of doubtful activity, 10 to 100 mgms. being without effect on frogs, while similar doses caused only a trifling rise of blood-pressure in mammals, without convulsions or other symptoms. Similarly Meulenhoff concluded that the activity of the crystalline ergotinine represented only a very small part of that of the ergot containing it.

The results of our own experiments point in the same direction. We have examined commercial specimens of ergotinine obtained from two well-known German firms. The specimens were described

1. Tanret distinguishes amorphous and crystalline ergotinine. It will be seen that we regard the so-called amorphous ergotinine as chemically different from the crystalline and as, in any case, impure. When, therefore, we use the term 'ergotinine' without qualification we mean the pure, crystalline alkaloid.

as crystalline, but contained at least 50 per cent. of an amorphous, greyish-green impurity, which was readily soluble in warm alcohol, and was strongly active physiologically. On recrystallising the part which was less readily soluble, from boiling alcohol, we obtained a rather small yield of white needles corresponding in all respects to Tanret's description of crystalline ergotinine.

The ergotinine thus obtained from the commercial specimens, and various other specimens of pure ergotinine prepared by ourselves, showed, on intravenous injection into cats, a very variable degree of activity. In two or three instances a complete vaso-motor reversal was obtained with doses of 1.5 to 2 mgms. per kilo. of body weight (as compared with 0.5 mgm. of ergotoxine). In most cases, however, a dose of 4 to 6 mgms. per kilo. produced only a slight rise of blood-pressure and a mere indication of the vaso-motor reversal; in a few cases there was no trace of either action. At first we were at a loss to explain these irregularities. All the specimens were well crystallised, and their purity was established as far as possible by analysis and by physical tests. We were led to the assumption that, according to the method used for putting the ergotinine into solution, a greater or smaller portion was decomposed, forming a highly active substance. Sometimes we dissolved the ergotinine in the minimum quantity of boiling alcohol (about 0.8 c.c. for 10 mgms.), added one or two drops of 10 per cent. caustic soda solution, and could then dilute with water. In other cases a little more than the theoretical quantity of phosphoric acid was added to the alcoholic ergotinine solution, which could then be diluted with water to a certain extent. We also dissolved the alkaloid in the minimum quantity of glacial acetic acid. Any attempt to neutralise the excess of acid, however, caused precipitation of the ergotinine salt.

After having found that a solution of ergotinine in caustic soda became somewhat more active on boiling, we investigated the action of phosphoric acid, hoping to produce the characteristic ergotoxine phosphate.

0.3 gramme of ergotinine was boiled for one hour with 10 c.c. of absolute alcohol, with a reflux condenser. As was expected, a considerable portion remained undissolved.



0.6 c.c. of 10 per cent. aqueous phosphoric acid solution, corresponding approximately to 1.2 molecular equivalents, was now added, and after five to ten minutes' further boiling solution was complete. On cooling, crystallisation was induced by scratching with a glass rod; the first crop of crystals weighed 0.07 gramme (= 25 per cent.), and consisted of minute diamond-shaped plates, melting at  $182^{\circ}$ . The thin prisms of ergotinine were entirely absent. As ergotoxine phosphate, crystallising in prisms, melts at  $182^{\circ}$  to  $183^{\circ}$ , and as the melting point was not lowered after mixing with a quantity of the new plates, it seemed probable that the latter represented ergotoxine phosphate. In support of this view was also the fact that the new substance yielded, on decomposition with ammonia, an amorphous base apparently identical with ergotoxine.

The new phosphate thus obtained from ergotinine was tested physiologically by intravenous injection into a cat. In the first place 3 mgms. of the same ergotinine specimen as that which had been submitted to hydrolysis were given, dissolved in 2.5 c.c. of 50 per cent. alcohol, mixed with a little saponin (to prevent crystallisation).

Then 2 mgms. of the new phosphate were injected, dissolved in a little dilute alcohol, to which the same quantity of saponin had been added. The effect of the two injections is shown in Fig. 7.

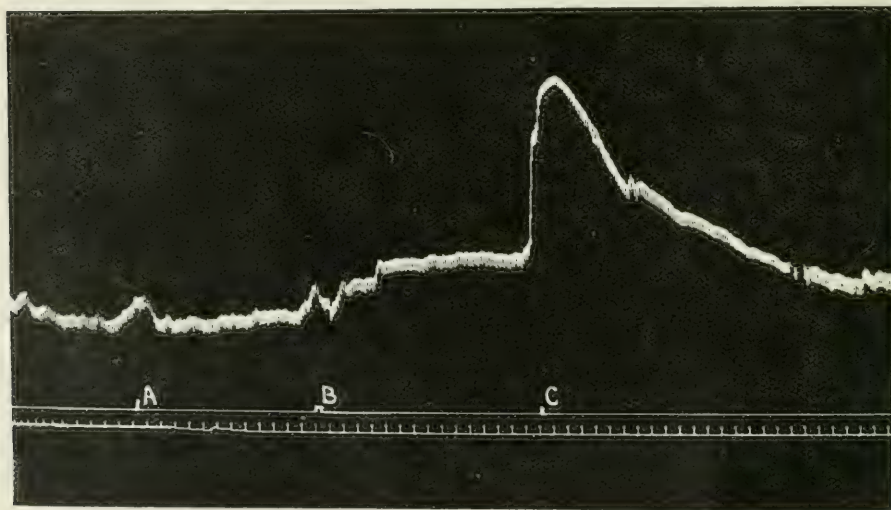


FIG. 7A

Cat,  $2\frac{1}{2}$  kilos. Pithed. Artificial respiration. Carotid blood-pressure. Injections into jugular vein.

Effects of ergotinine—

At A—1 mgm. of ergotinine.

At B—3 mgms. of ergotinine.

At C—0.05 mgm. of the suprarenal principle.

The ergotinine produces little pressor effect, and no subsequent vaso-motor reversal.



As will be seen, the ergotinine produced a small rise of blood-pressure, and practically no vaso-motor reversal, whereas a smaller quantity of the phosphate of the amorphous alkaloid, obtained from it, caused a very marked rise of blood-pressure and complete vaso-motor reversal.

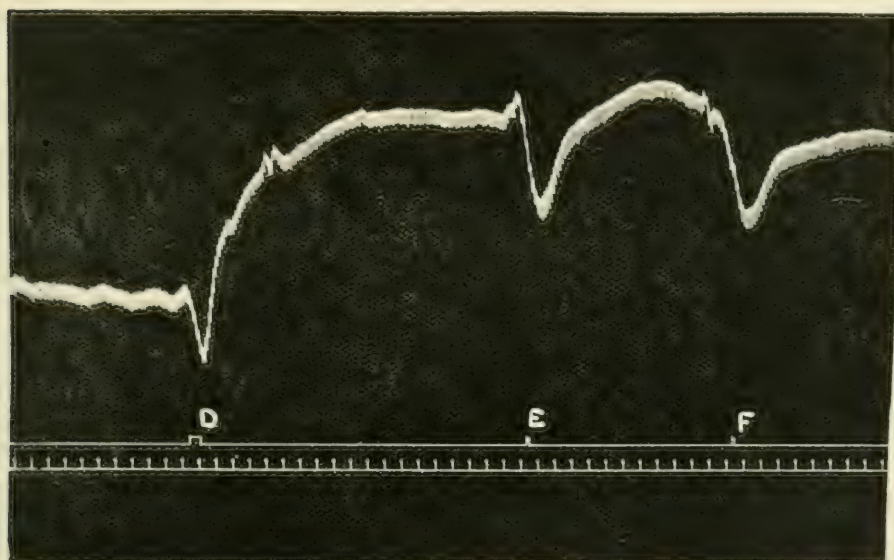


FIG. 7B

*Same animal as in Fig. 7A*

Subsequent effect of crystalline phosphate (ergotoxine —

At D—2 mgms. of the crystalline phosphate.

At E—0.05 mgm. of the suprarenal principle.

At F—0.1 mgm. of the suprarenal principle.

The ergotoxine gives a good pressor effect and complete vaso-motor reversal.

We had previously obtained the same crystalline (ergotoxine) phosphate in an attempt to crystallise the phosphate of ergotinine. 1.15 grammes of ergotinine (crystallised from alcohol) was dissolved in a mixture of alcohol and ethyl acetate and 2.5 c.c. of 10 per cent. phosphoric acid were added. Accepting a wrong value for the molecular weight of ergotinine, we believed this quantity of acid to be equal to one molecular equivalent. In reality there was one and a quarter molecules. The solution of the phosphate was concentrated in a vacuum dessicator at 37°, and the amorphous substance which separated out (ergotinine phosphate?) was filtered off. It was easily soluble in alcohol on warming, and on cooling it again separated out amorphous. Finally, however, on concentrating the mother liquor of the amorphous substance, we obtained a minute quantity of imperfectly formed

plates, which consisted of the phosphate of an amorphous base, and of which 1 mgm., dissolved in 0.5 c.c. of 50 per cent. alcohol produced complete vaso-motor reversal in a cat of 2.7 kilos.

This result, though somewhat puzzling at the time, is now perfectly intelligible in the light of our later knowledge.

These experiments make it clear that ergotinine is certainly much less active than ergotoxine, and make it, indeed, very doubtful whether as such it has any activity at all. It is true that the alkaloid, when separated in the pure condition, is a particularly unsuitable substance for physiological experiments. If, on the one hand, it is injected into the blood-stream in some neutral solvent, such as alcohol, the whole of the alkaloid separates in an insoluble form as soon as the solution comes into contact with the blood. If, on the other hand, it is dissolved for injection by the aid of acids or alkalis, not only does a considerable proportion of the feebly acidic and basic ergotinine separate out on dilution with the blood, but one cannot exclude, under such conditions, the possibility of ergotoxine formation. It may be conceded, therefore, that, if ergotinine could be brought into solution without risk of conversion into ergotoxine, and could remain dissolved in the body fluids, it might exhibit physiological activity, even when injected intravenously. If it is given hypodermically the possibility of slow conversion into ergotoxine in the subcutaneous tissues gives to ergotinine a further chance of showing itself physiologically active. This may, in part, explain the toxic effect of ergotinine on guinea-pigs, in the experiments recorded by Kraft. However that may be, our own experiments confirm those of Kobert and Meulenhoff, and lead to the conclusion that, whether because of its insolubility or otherwise, the activity of ergotinine is negligible in comparison with that of ergotoxine.

In support of this conclusion we may also cite Jacobj's experience (37) with the inactive alkaloid, which he named 'secaline,' but which can, beyond reasonable doubt, be identified with ergotinine.

Jacobj's reasons for regarding it as a new alkaloid were to a large extent imaginary, and founded on misconceptions. The first of these was as follows :—The alkaloid gave the colour reaction found by Keller to be characteristic of cornutine; but it could not

be cornutine, which Kobert described as of great physiological activity, for Jacobj's alkaloid was physiologically inert, resembling in this respect the ergotinine of Tanret, which, on Kobert's authority, was devoid of activity. In other words, Jacobj's alkaloid resembled Tanret's ergotinine in its inactivity, but gave the colour reaction attributed by Keller to cornutine. The explanation is simple. The whole confusion is due to the fact that Keller, at the time when he described his reaction, regarded the two alkaloids as one, and selected for that one the name cornutine. On this ground, therefore, the evidence is all in favour of the identity of secaline and ergotinine.

Another of Jacobj's reasons for concluding against this identity was a chemical one. He found on analysis 49.01 per cent. of carbon and 11.62 per cent. of nitrogen, whereas Tanret had found in ergotinine 68.62 per cent. of carbon and 9.15 per cent. of nitrogen. The discrepancy in the nitrogen figures is explained by the fact that Tanret used the unreliable soda-lime method and that his figure was erroneous, as was shown by one of us in conjunction with F. H. Carr (51). The mean of four analyses according to Dumas gave 11.6 per cent. of nitrogen, a figure which Tanret himself has recently (49) confirmed. As will be seen, the agreement with the value given by Jacobj for secaline is excellent. We cannot explain the discrepancy of 20 per cent. between Jacobj's percentage of carbon and the figures found by Tanret and by Barger and Carr, but must be content to point out that Jacobj performed only one combustion, and that of an amorphous substance, the purity of which he deduced from its white colour and physiological inactivity. It is interesting to note that on repeating the preparation of secaline, Jacobj could not 'for some unknown reason' obtain a similarly inactive preparation. We may safely assume that this unknown reason was the presence of ergotoxine, which cannot be separated from ergotinine by precipitation of the ethereal solution with light petroleum, the method employed by Jacobj.

### (c) *Cornutine*

In an earlier paper one of us (43) applied the name cornutine to an alkaloidal substance (which we now know to have been impure ergotoxine), on the ground that this substance resembled in solubility cornutine more than any other of the active substances hitherto described in ergot. We were anxious, moreover, to avoid 'the introduction of new names on the strength of physiological results, and in default of the chemical isolation of principles.' When this amorphous alkaloid had been obtained in a pure form it was found to differ not only in chemical properties from cornutine, but also in those physiological properties which, according to Kobert, constituted the real claim of cornutine to recognition as a separate substance. Among the few chemical characteristics of cornutine, which were



mentioned by Kobert, are insolubility in ether and the possession of a hydrochloride which was readily soluble in water. Our alkaloid is, like ergotinine, slightly but distinctly soluble in ether, and its hydrochloride is remarkably insoluble in water. From the physiological point of view, which is of primary importance in the case of a substance for which only 'physiological purity' is claimed, our alkaloid also showed very marked differences from cornutine. While resembling both sphacelinic acid and cornutine in causing rise of blood-pressure and contraction of the uterus, it differed from cornutine in both the properties which Kobert regarded as characteristic of that substance; it did not cause the strychnine-like spasms in frogs, and it did cause gangrene of the cock's comb. On the whole the physiological effect of the alkaloid was much more like that of sphacelinic acid than like that of cornutine. For a pure substance presenting such confusing resemblances to several 'active principles,' yet equally confusing differences from any one in particular, the choice of a new name was inevitable, and the name ergotoxine was accordingly suggested for it by Barger and Carr (45) in their chemical account of the substance.

There is, however, good reason for believing that cornutine contains some ergotoxine. According to Tanret (33), cornutine gives (feebly) the sulphuric acid colour reaction for ergotinine, which is also given, as we have pointed out, by ergotoxine. Specimens of cornutine, which we prepared according to Kobert's method, gave, on intravenous injection into cats, a considerable rise of blood-pressure, with some small subsequent indication of a vaso-motor reversal.

In addition to ergotoxine, it is probable that cornutine contains decomposition products of the former, and it is possible that these decomposition products are concerned in the strong convulsant action on frogs. It is noteworthy, however, that other observers, such as Meulenhoff, have failed to produce the convulsions in frogs with cornutine prepared according to Kobert's method. We may remark in passing that this method, which begins with an extraction of the ergot by dilute hydrochloric acid, is scarcely a suitable one for the



removal of the ergotinine and ergotoxine, since their chlorides are very little soluble in dilute hydrochloric acid, so that the yield of cornutine is necessarily very small.

As we have pointed out above, Kobert's term cornutine was applied later by Keller to ergotinine. 'Cornutin-Keller,' prepared by Keller and others, was examined physiologically by Santesson (39), who remarks that it is not identical with Kobert's cornutine, and that it is apparently less active. Santesson describes his preparations as impure; all but one were partially crystalline. Thirteen to twenty-five milligrammes injected hypodermically produced a violet coloration of the cock's comb, but true gangrene was never observed. In rabbits a dose of 10 to 16 mgms. per kilo., given hypodermically, produced no pronounced effect; 40 mgms. per kilo. caused a non-fatal intoxication. Intravenously 5 mgms. produced a marked rise of blood-pressure in the cock, but in rabbits only a small and transitory rise was occasionally obtained. Santesson inclines to the view that 'cornutin-Keller' is not the chief active principle in ergot, and that it is probably identical with the ergotinine of Tanret. From Santesson's physiological results and Keller's method of preparation it seems likely that the specimens employed were mixtures of ergotinine and ergotoxine, containing perhaps something like 25 per cent. of the latter alkaloid.

Santesson's failure to obtain a rise of blood-pressure in the rabbit in no way excludes the presence of ergotoxine in his preparations, for, as we have observed, rodents are especially insensitive to the stimulant action of ergotoxine, and in particular show but a slight vaso-constrictor effect. Moreover, his animals were not given artificial respiration, without which, as we have pointed out, the pressor effect is not well shown even in so responsive an animal as the cat.

THE PRESENCE OF ERGOTOXINE IN SUPPOSED ACTIVE PRINCIPLES OF  
A NON-ALKALOIDAL NATURE

The feebly basic nature of ergotoxine, its acidic character, due to a phenolic hydroxyl, the colloidal condition of its salts in aqueous solution, its large molecular weight and its amorphous nature—all these properties may be held responsible for the fact that ergotoxine clings so tenaciously to the resins, fat, and colouring matters present in ergot, and have determined its presence in the various non-alkaloidal substances described as active principles by Kobert, Jacobj, and Meulenhoff. Of these Jacobj's chrysotoxin is the most clearly characterised; on this account, and because it was the starting point of our own investigation, it will be convenient to deal first with the work of this observer.

*(a) Jacobj's Preparations*

Theoretically there is something to be said for the use of indifferent solvents of low boiling point, such as ether and light petroleum, and Jacobj (37) in so far achieved his object, that the decomposition of the active substance was probably less in his than, for instance, in Kobert's experiments. On the other hand, it is impossible to separate the active substance from inert constituents by the use of such solvents alone.

There are two ways of preparing the fat-free ethereal extract, which corresponds to Jacobj's crude chrysotoxin. In the first place the ergot can be extracted with ether; the ether is then evaporated, and a large volume of light petroleum is added, which precipitates the 'chrysotoxin,' and leaves the oil in solution. Secondly, the ergot can be extracted first with light petroleum, which only removes the oil, and then with ether. Fatty oil is present in ergot to the extent of about 33 per cent., but the amount which is readily extracted by percolation with light petroleum amounted, in our experiments, only to about 25 per cent. of the weight of the ergot. Kraft also found that at least 5 per cent. of oil remained in ergot after the most careful extraction with light petroleum.

On this account we chose, as did Kraft, the first method, viz., extraction of the ergot with ether, and subsequent precipitation with light petroleum. We can confirm Kraft's statement that a thorough extraction with ether is difficult to achieve. 1 kilo. of ergot,

in No. 40 powder, was percolated with 10 to 12 litres of dry ether. Nearly all the oil was removed by the first few litres of the percolate. On evaporating this, finally in vacuo, there remained 350 c.c. of oil, which, when mixed with light petroleum (700 c.c.), gave a precipitate of a grey solid, weighing 0.36 gramme. This produced the vaso-motor reversal in a dose of 15 mgms. per kilo. of cat. From the solution of the oil in light petroleum 0.060 gramme of ergotinine was obtained by shaking with acids.

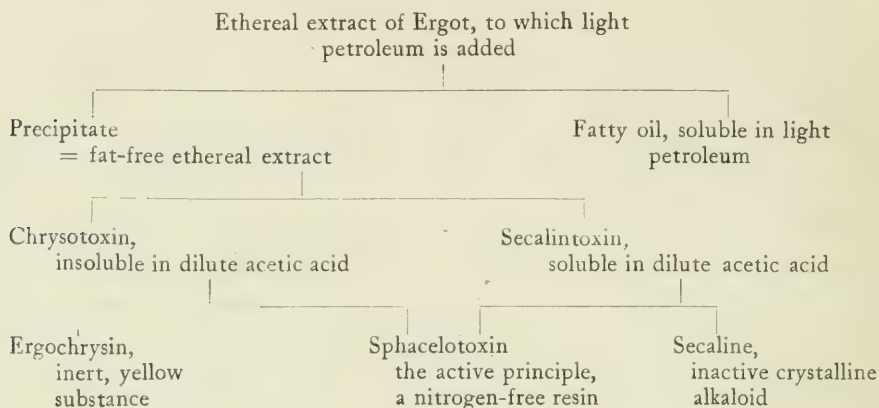
On continued percolation with ether, a yellowish-brown solid was obtained, which, after washing with light petroleum, weighed 0.156 gramme. The last three litres of the percolate together only left 0.013 gramme of a solid, which was very slightly soluble in ether. In all we had obtained 0.19 per cent. of the weight of the ergot as fat-free ether extract, but as the extraction proceeded so slowly and was incomplete, the experiment was abandoned.

Extraction with ether in a Soxhlet apparatus likewise gave bad results. 30 grammes of the same ergot powder were extracted for three weeks. The extract, after treatment with light petroleum, yielded 0.07 gramme (0.23 per cent.) of a dark brown solid, which was tested physiologically. The vaso-motor reversal was nearly produced by a dose of 2.8 mgms. per kilo., and was complete after 5.6 mgms. We may put the dose at roughly 4 mgms. per kilo., corresponding to 1.6 grammes of ergot.

The ether-extracted powder in the Soxhlet apparatus was next exhausted for six days with 90 per cent. alcohol. A dose of the alcoholic extract corresponding to 4 grammes of ergot per kilo. of cat was necessary to produce the vaso-motor reversal. Assuming that all the active substance had now been extracted from the drug, we may conclude that the amount removed by ether in a Soxhlet apparatus was  $\frac{4}{1.6} = 2\frac{1}{2}$  times that which had been left in the ergot, so that after three weeks' extraction something like two-sevenths of the substance still remained behind. The only satisfactory method of ether-extraction seems to be the process employed by Kraft, based on Keller's assay method for ergot. Kraft shook the moistened ergot powder with ether for an hour at a time and repeated this ten times with fresh quantities of ether, thus obtaining 0.5 per cent. of fat-free extract and almost complete exhaustion (as compared with 0.19 and 0.23 per cent., which we obtained in a percolator and a Soxhlet apparatus respectively). In the light of these results we can readily understand Meulenhoff's conclusion, that simple percolation with ether removes very little of the active substance. He found that 2 kilos. of ergot, after percolation with 5 kilos. of ether, lost less than 20 per cent. of the active substance, as measured by toxicity to cocks. We also examined the solvent action of acetone on ergot. It resembles that of ether more than that of alcohol. By percolation with acetone and subsequent treatment of the extract with light petroleum we readily got 0.3 per cent. of a fat-free extract, closely resembling the extract obtained more slowly with ether.

According to Jacobj (37) the fat-free ether extract contains non-basic chrysotoxin and alkaloidal secalintoxin, both compounds of the active principle sphacelotoxin with the inert substances, ergochrysin

and secaline respectively. His conception of the relationship of these substances will be clear from the following diagram :—



Ergochrysin sphacelotoxin, and, therefore, also chrysotoxin, are, according to Jacobj, non-nitrogenous. Chrysotoxin in doses of 0.1 gramme produced the characteristic violet coloration of the cock's comb. The alkaloid secalintoxin had the same effect, but was about five times as potent. By repeated extraction with acetic acid chrysotoxin was changed to ergochrysin, which had the same chemical properties, but was physiologically inert. Hence, according to Jacobj, chrysotoxin must be a *compound* of ergochrysin and the active principle sphacelotoxin. Secalintoxin was obtained from crude chrysotoxin by extraction with acid, and was itself separated into the crystalline alkaloid secaline and the non-nitrogenous resin sphacelotoxin by mere treatment with neutral organic solvents. It hardly needs pointing out that neither chrysotoxin nor secalintoxin had any real claim to be considered a chemical compound. We have already given reasons for identifying secaline with Tanret's ergotinine; ergochrysin being admittedly inactive, does not directly concern us: the important point is the nature of sphacelotoxin.

Using the vaso-motor reversal as a measure of activity, we, therefore, repeated Jacobj's experiments.

A quantity of crude 'chrysotoxin' was purified by fractional precipitation of its solution in ether by light petroleum. It was a



yellow powder very soluble in ether. Several specimens were converted into the water-soluble sodium-compound, by the addition of an alcoholic solution of sodium ethylate to the ethereal chrysotoxin-solution, carefully avoiding excess.

As this process was always accompanied by a varying loss of activity, we later preferred to dissolve the substances, for intravenous injection, in the minimum quantity of alcohol; adding one or two drops of caustic soda we could then dilute indefinitely with water. The dose of chrysotoxin necessary to produce the vaso-motor reversal was rather variable, but, in our chief preparation, amounted to about 0.025 gramme per kilo. of cat.

We next dissolved the chrysotoxin in glacial acetic acid, and poured the solution into water. On filtering, the solution contained most of the active substance and gave alkaloidal reactions, but the precipitate was still distinctly active, and the separation was not sharp. Thinking that acetic acid was not a sufficiently strong acid, we next dissolved the chrysotoxin in the minimum quantity of alcohol, and poured the solution into 5 per cent. aqueous sulphuric acid.<sup>1</sup> The precipitate was collected and treated in the same way. After this process had been performed three times the activity of the precipitate had fallen to about one-seventh of its original value, since 0.170 gramme per kilo. was now required to produce the vaso-motor reversal in a cat, instead of 0.025 of the original preparation. Continued treatment with sulphuric acid still further reduced the activity of the precipitate.

All the acid filtrates were mixed and made alkaline with sodium carbonate. In this way a white flocculent precipitate of alkaloids was obtained, soluble in caustic soda and constituting about 1 per cent. of the chrysotoxin employed. The alkaloidal precipitate was found to contain a considerable part of the activity of the original chrysotoxin, a dose corresponding to 0.07 gramme of chrysotoxin per kilo. producing the vaso-motor reversal. The mixture of alkaloids thus obtained obviously corresponded to Jacobj's 'secalintoxin.' When its ethereal solution was evaporated it deposited crystals of ergotinine (= secaline), which were identified by the melting point (219°). The mother liquor left behind an amorphous alkaloid closely resembling the pure alkaloid subsequently named ergotoxine, and at the same time corresponding to Jacobj's 'sphacelotoxin.' The same separation of the alkaloidal constituents from chrysotoxin was per-

1. Kraft (46) has since shown that the sulphate of ergotoxine (hydroergotinine) is particularly insoluble, a fact which, no doubt, in part accounts for our comparative failure to free the chrysotoxin from active alkaloid.

formed with great care by Kraft, using glacial acetic acid. After four successive precipitations with water, he found that the precipitate still contained traces of alkaloid, just as we found that it retained traces of physiological activity. The only consideration, therefore, which should make us hesitate to conclude that sphacelotoxin was merely an impure form of our alkaloid ergotoxine is Jacobj's statement that it contained no nitrogen. It must be remembered, however, that he never obtained his sphacelotoxin completely free from nitrogen, that he used but small quantities, and that he used sodium instead of potassium for Lassaigne's test. It is difficult to understand why he failed to apply a reagent immeasurably more sensitive, such as potassium mercuric iodide, which detects one part of ergotoxine in 2,000,000 parts of water. Similarly, by the use of alkaloidal precipitants, Jacobj might have detected the presence of alkaloids in active specimens of chrysotoxin. In any case, his failure to detect nitrogen qualitatively can be of no significance when we consider his quantitative results. Two determinations gave him for the mixture of alkaloids, called secalintoxin, 11.71 and 11.79 per cent. of nitrogen. In the one constituent, secaline, he found 11.62 per cent.; obviously, therefore, the other constituent, sphacelotoxin, must have had about the same nitrogen content. Compare with this the nitrogen contents of ergotinine, 11.5 per cent., and of ergotoxine, 11.2 per cent. (Barger and Carr). Similarly the carbon content of secalintoxin was but slightly below that of ergotinine and of ergotoxine. Apart from the supposed absence of nitrogen, sphacelotoxin corresponds in all characters, chemical and physiological, to an impure and partially decomposed mixture containing probably up to 50 per cent. of ergotoxine. Jacobj records a marked effect on the cock with 5 and with 8 mgms. sphacelotoxin; we obtained a very marked and typical effect with 2 mgms. of ergotoxine (see p. 266). The great solubility in alcohol and the green colour developed on standing are both characteristic of ergotoxine, while the yellow colour of fresh preparations is due to a tenaciously adhering colouring matter. Jacobj's partial success in separating sphacelotoxin from secaline as a lead salt corresponds to the separation of ergotoxine from ergotinine by dilute caustic soda (Barger and Carr).

We conclude, therefore, that the only active substance present in Jacoby's preparations was the alkaloid ergotoxine; that this, in relatively pure form, constituted his sphacelotoxin; that, mixed with ergotinine, it was present in secalintoxin; and that it occurred as a contamination to the extent of about 2 per cent. in chrysotoxin.

(b) *The Sphacelinic Acid of Kobert*

Jacoby regarded chrysotoxin as the sphacelinic acid of Kobert (20) in a pure form. He found that a specimen of this substance, prepared according to the method of Kobert and Bombelon, consisted for two-thirds of an inert fatty substance soluble in light petroleum, and, for the rest, of an active substance, which resembled chrysotoxin that had been decomposed by alkali.

Our view that Jacoby's chrysotoxin owed its activity to contamination with an active alkaloid (ergotoxine) applies equally to Kobert's sphacelinic acid. We put this view to the test of experiment, and prepared sphacelinic acid according to Kobert's original method.

Ergot powder was percolated successively with light petroleum, ether, and 90 per cent. alcohol. The residue left on evaporation of the alcoholic percolate was extracted first with water, and then with phosphoric acid. The extraction with phosphoric acid at this stage constitutes a slight departure from Kobert's method, and replaces his preliminary extraction of the ergot with 3 per cent. hydrochloric acid. As the chlorides of the ergot alkaloids are very difficultly soluble, we considered that our variation of Kobert's method would be more effective in removing the alkaloids. The brown resin which remained behind after extraction with phosphoric acid was finally dissolved in caustic soda, and precipitated by phosphoric acid. In this way we obtained from 500 grammes of ergot 0.6 gramme of an almost fat-free greyish-brown powder, showing in a high degree the physiological activity characteristic of ergotoxine. A dose of 3 mgms. per kilo. was more than sufficient to produce a complete vaso-motor reversal in the cat.

The preparation was about as active in this respect as the most active chrysotoxin which we had prepared.

This statement scarcely agrees with Jacoby's view of chrysotoxin as a purer form of sphacelinic acid—at least, if by 'purer' we mean richer in activity. On the other hand, chrysotoxin contains the inert yellow colouring-matter in a purer form than sphacelinic acid. Compared with the sphacelinic acid used by Grünfeld and by Jacoby



(the latter's specimen was two-thirds fat) our preparation was presumably much more active.

In order to prove that our sphacelinic acid owed its activity to an alkaloidal contamination, a solution of 0.5 grammes in dilute caustic soda was poured into 100 c.c. of 10 per cent. phosphoric acid. The precipitate was filtered off at the pump, and washed with water; it was then redissolved in a little caustic soda, and again poured into acid. This process was gone through three times. The filtrates, which were quite clear, gave alkaloidal reactions, but not very strongly. By making alkaline with sodium carbonate and shaking out with chloroform we collected the minute quantity of alkaloid, and injected a dose equivalent to 10 mgms. of the original sphacelinic acid into a cat of 3 kilos. A very distinct ergotoxine effect was produced, but the vaso-motor reversal was incomplete. We then gave a corresponding dose of the sphacelinic acid which had been extracted with phosphoric acid. It also showed activity, though it was much less active than the original preparation.

At first we were at a loss to explain the fact that by methods calculated, as we believed, to remove the whole of the alkaloid, we could not remove the whole of the ergotoxine-activity, but a study of the properties of pure ergotoxine salts afforded us an explanation. The phosphate of ergotoxine, for instance, can form a typically colloidal solution in water, containing as much as 1 per cent. Like many other colloids it is, however, precipitated by any well-ionized electrolyte; therefore by all salts and strong mineral acids, but not by the weaker acids. Phosphoric acid alone does not precipitate the ergotoxine phosphate, so that the alkaloid might have been extracted, but the sodium phosphate formed inevitably precipitated much of the ergotoxine phosphate on to the sphacelinic acid. In the presence of excess of sodium phosphate we have found the true solubility of ergotoxine phosphate to be of the order of 1 : 20,000. The three filtrates of rather more than 100 c.c. each might contain very roughly 10 mgms. of ergotoxine, and the dose administered would be  $\frac{1}{50}$ th of this, or something like  $\frac{1}{3}$ th mgm. For complete vaso-motor reversal (in the cat of 3 kilos.) about 1.5 mgm. of ergotoxine phosphate would be required.

In another somewhat similar experiment we were more successful in effecting a complete separation.



An alcoholic extract was freed from fat by means of light petroleum, and was then suspended in water. 50 c.c. of the mixture, corresponding to 100 grammes of ergot, were poured into 500 c.c. of 5 per cent. hydrochloric acid. The liquid was then filtered and the process repeated five times. The last precipitate was dissolved by means of a little caustic soda, and a dose equivalent to 10 grammes of ergot was injected intravenously into a cat. The only noticeable effect was a temporary depression of the heart's action. A dose of the alkaloid extracted from the six filtrates and also equivalent to 10 grammes of ergot was next given to the same cat, and produced a marked rise of blood-pressure, with subsequent complete vaso-motor reversal, so that even 1 mgm. of the suprarenal active principle produced a fall of blood-pressure.

In this way it was proved that the whole of the active principle producing the vaso-motor effects here in question has basic properties and can be removed by acids. Very instructive in this connection are experiments made by Grünfeld (30) under Kobert's direction with a preparation which he calls 'crude sphacelinic acid' (Rohsphacelinsäure). This substance, Grünfeld explains, was obtained in the purification of ergotinine of Tanret. The crude ergotinine was dissolved in chloroform and ether added until the portion remaining behind in the ether-chloroform solution was colourless. The resinous precipitate, produced by the addition of ether, was called sphacelinic acid, because of its action on the cock's comb, and apparently not for any chemical reason. Chemically speaking, it cannot be doubted that the precipitate contained a considerable quantity of ergotoxine, which, as has been shown, is but slightly soluble in ether. Grünfeld found that doses of 1, 1 and 0.6 gramme of the precipitate gave what he describes as 'a most characteristic picture of gangrenous ergot poisoning,' and adds that the effect was equivalent to that of 7.0 grammes of 'pure sphacelinic acid, which, moreover, was administered only six months after the harvest.'

This experiment from Kobert's own laboratory points to the conclusion that the true gangrenous effects on the cock's comb, like the vaso-motor effects on the cat in our own experiments, are produced by an alkaloidal and not by an acid constituent of ergot. In the light of our later experiments, described in a former section of this paper, it can now be definitely stated that this basic principle is the alkaloid ergotoxine, which in the pure state not only produces the

vaso-motor reversal in the cat, but also the gangrene of the cock's comb and other effects regarded by Kobert as characteristic of sphacelinic acid. The chain of evidence, therefore, seems complete for the conclusion that sphacelinic acid, like chrysotoxin, owes its activity to adherent ergotoxine.

#### THE PREPARATIONS OF THE BRITISH PHARMACOPOEIA

From the physical and chemical properties of ergotoxine already described, it will be clear that its occurrence in any considerable proportion in the official aqueous extract (*Extractum Ergotae Liquidum*) is not probable. Small traces of an alkaloid, soluble in chloroform, and giving the physiological reactions of ergotoxine, can be obtained from most specimens of the extract. Since ergot always contains a considerable quantity of di-acid potassium phosphate, these traces of ergotoxine are probably dissolved as the phosphate, which, in the presence of salts, is very slightly but distinctly soluble. In our experience, however, the stimulant effect of the liquid extract on involuntary muscle, as indicated by its power of raising the blood-pressure, is much too great to be accounted for by the amount of ergotoxine present, as indicated by the vaso-motor reversal, and by the amount which can be extracted by chloroform. The same discrepancy appears to have been met with by Cushny (50) in his experiments on the effect of ergot extracts on the uterus. With regard to the pressor effect, Dixon (42) has pointed out that liquid extracts have a marked augmentor effect on the action of the heart, and the disproportion between the rise of blood-pressure and the amount of ergotoxine present might, in part, be accounted for by the presence of some cardiac stimulant principle. According to Plumier (40), the fluid extract which he examined contained a principle which caused rise of both aortic and pulmonary pressure, due to constriction of both systemic and pulmonary arterioles, the effect of 'ergotinine' being smaller, and confined to the systemic vessels. Recently Meltzer and Auer (48) have described an effect of the fluid extract (U.S.P.) on the movements of the stomach and intestines.

They regard the augmentation of movements and increased sensitiveness to vagus-stimulation which they observed as similar to the effects obtained by one of us (43) with chrysotoxin and other ergotoxine-containing preparations. We have no indication as to the richness in ergotoxine of the particular fluid extract which they used, but, from general experience of such preparations, we are disposed to regard it as doubtful whether the phenomena which they observed are due to ergotoxine at all. Especially significant is their insistence on the striking increase of gastro-intestinal movement, following injections of the extract. We can only confirm the statement in the former paper, that the effect, in this direction, of ergotoxine, and of preparations owing their activity entirely to it, is comparatively slight and inconstant. The effect on intestinal movements of a complex fluid such as the liquid extract, containing, apart from principles the action of which is peculiar to ergot, choline and various other vascular depressants (ergotinic acid, etc.), seems to us to need a more critical analysis before any great importance is attached to it as a specific action.

The same criticism applies more obviously to the numerous descriptions of a fall of blood-pressure as the characteristic effect of injecting ergot preparations. (Cf. Sollmann and Brown (41), who give references to other similar papers.)

In regard to the amount of ergotoxine present, the position of the *Extractum Ergotae* ('Ergotin') of the British Pharmacopoeia is not widely different from that of the liquid extract. Although the ergot is, in the first place, extracted with 60 per cent. alcohol, nearly the whole of the alkaloid so extracted is subsequently removed in the resin which is precipitated by the addition of hydrochloric acid after removal of the alcohol. It may, indeed, be said that the ergotoxine-content of 'ergotin' is in inverse relation to the care with which the official instructions are carried out. Like the liquid extract, carefully made specimens of the official ergotin appear to have a more marked pressor effect than is accounted for by the small amount of ergotoxine present, though the question of the existence of a second stimulant principle must in our opinion be regarded as not finally decided until it can be separated from the physiologically active substances.



Of the remaining pharmacopoeial preparations, the infusion does not require consideration apart from the liquid extract, and the *injectio hypodermica* is merely a solution of ergotin. The ammoniated tincture contains, as might be expected, a larger proportion of ergotoxine than any other official preparation, as indicated by the physiological test. It does not, however, contain by any means the whole of the ergotoxine of the ergot from which it is prepared. It is doubtful whether there is any advantage in the use of ammonia in the extraction, and its presence in the tincture may facilitate the decomposition of the ergotoxine.

#### VAHLEN'S CLAVIN

In considering the possibility that pharmacopoeial preparations, such as the liquid extract, may contain an active principle distinct from ergotoxine, Vahlen's recent claim (44) to have isolated a water-soluble active principle is of interest. Starting from the discrepancy between the extensive clinical use of the liquid extract and the fact that all the 'active principles' hitherto prepared by pharmacologists were insoluble in water, Vahlen succeeded in obtaining from watery extracts a crystalline neutral substance. He stated that the substance clavin produced, in the pregnant animal, a co-ordinated peristaltic activity of the uterus of the type seen in normal labour, but that it was devoid of any toxic properties, and was thereby sharply differentiated from other active principles of ergot.

As soon as clavin was commercially obtainable we submitted a sample to physiological experiment. The animals used were cats, pregnant and non-pregnant, and rabbits, of which one was in the latest stage of pregnancy. The conditions observed by Vahlen himself have in several instances been reproduced, the animal being immersed in a saline bath at  $37^{\circ}$ , and anaesthesia being produced by urethane. In all, three commercial specimens, obtained at different times, were used, and, in addition, a specimen prepared in the laboratory by ourselves. In no case was any trace observed of the action described by Vahlen; a temporary slowing of the respiration and, in one case, a slight and evanescent depression of the heart's action



were the only effects observed. This last effect was accounted for by the presence of acid potassium phosphate, with which this specimen was contaminated. A similar absence of effect of clavin on the uterus has been observed by Cushny (50).

The following is the complete record of one of our experiments, made on a cat in advanced pregnancy :—

CAT, weight 3 kilos. Anaesthetised by injection of 4·5 grammes of urethane. The blood-pressure was recorded from the carotid artery, and injections made into the external jugular vein. The abdominal wall was not opened until the abdomen had been submerged in a bath of physiological saline at 37°.

11.50 a.m. Abdominal wall opened in the bath of warm saline. Uterus, containing two foetal sacs in each horn, was flaccid and inactive. It remained so till

11.55 a.m., when it contracted for twenty to twenty-five seconds, and then relaxed again.

The contraction affected both horns equally, and appeared uniform in distribution.

11.59 a.m. A similar contraction, again lasting twenty to twenty-five seconds.

12.2 p.m. Contraction for thirty seconds.

12.6 p.m. Contraction for thirty to forty-five seconds.

12.7 to 12.9 p.m. Injection of 70 mgms. of clavin, dissolved in 7 c.c. of physiological saline, into the jugular vein.

12.10 p.m. Contraction of the uterus, lasting rather more than thirty seconds.

12.14½ p.m. Contraction for rather more than one minute.

12.17 p.m. Contraction for about thirty seconds.

12.18 p.m. Contraction for about ten seconds.

12.19 to 12.20 p.m. Ether given by inhalation.

12.22 p.m. Contraction of uterus for twenty seconds.

12.22½ p.m. Contraction for fifteen seconds.

12.23 p.m. Ether for one minute.

12.24 p.m. Contraction for twenty seconds.

12.25 p.m. Contraction for fifteen seconds.

12.25½ p.m. Contraction for twenty seconds.

12.27 p.m. Contraction for twenty seconds.

12.29 p.m. Contraction for twenty seconds.

Beside these larger contractions, small irregular waves of contraction have all along been seen passing over the uterus. The main contractions have been, since the injection of clavin, distinctly less powerful than before, though rather more frequent. A second injection of 80 mgms. of clavin was given at 12.38 p.m., and the uterus watched till 12.59 p.m., with the result that no alteration in its activity was detected. The same normal, rhythmic contraction of the organ proceeded at intervals of one to three minutes, each lasting for twenty to thirty seconds.

- 1.0 p.m. Injection of 2 mgms. of ergotoxine phosphate, in 1 c.c. of distilled water, into the jugular vein. Breathing becomes feeble and blood-pressure tends to fall as respiration stops. Artificial respiration given.
- 1.1 to 1.2 p.m. Contractions of the normal type occurred.
- 1.3 p.m. Strong contraction, very marked between the foetal sacs.
- 1.5 p.m. Uterus still contracting, strongly between the foetal sacs.
- 1.7½ p.m. Strong rings of contraction, originating between and passing over the sacs towards the body of the uterus. The whole organ is tense and pale.
- 1.12 p.m. The waves of contraction continue. The tone of the whole organ seems slightly less.
- 1.15 p.m. Contraction continues.
- 1.15½ p.m. Injection of 0.1 mgm. of the suprarenal active principle intravenously.
- 1.16 p.m. Marked relaxation of the uterus, the outlines of the foetal sacs, which had been sharply defined by the constrictions between them, becoming far less apparent.
- 1.17 p.m. Reappearance of the tonic contraction.
- 1.18 p.m. Peristalsis recommencing.
- 1.35 p.m. Fairly vigorous peristalsis still continues. Cat killed.

In this experiment, therefore, 150 mgms. of clavin failed to produce any significant effect on the uterus, the slight weakening and acceleration of the normal rhythm being possibly due to the exposure in the saline bath, and, in any case, of no importance. Subsequently 2 mgms. of ergotoxine produced tone and marked peristaltic activity in the uterus, which, at that time, had already been exposed to the saline for over one hour.

Fig. 8 shows the effect of clavin and ergotoxine on the graphically recorded contractions of the uterus of another cat in early pregnancy.

Having failed to produce any physiological effect with clavin, and having satisfied ourselves that our specimens corresponded chemically to Vahlen's description, we proceeded to a more thorough investigation of its nature, and prepared a larger quantity by a slight modification of Vahlen's method.

22 kilos. of ergot were extracted with water; the extract was concentrated to 22 litres, and precipitated with 1500 grammes of barium hydrate; the precipitate was washed with 13 litres of water. On removal of the excess of baryta from the filtrate and washings, by means of sulphuric acid (about 50 grammes of barium sulphate being formed), the solution was concentrated *in vacuo* to a small bulk, mixed with 10 kilos. of silver sand, and dried completely *in vacuo* at 100°. The resulting product was extracted three times with 92 per cent. alcohol, and the resulting alcoholic solution (about 30 litres)

was evaporated. The dark brown residue was then dissolved in the minimum quantity of boiling 75 per cent. alcohol (3 litres); on standing a crystalline deposit was formed, which was recrystallised twice from 75 per cent. alcohol. The yield was 15 grammes, or about 0.07 per cent. of the ergot employed.

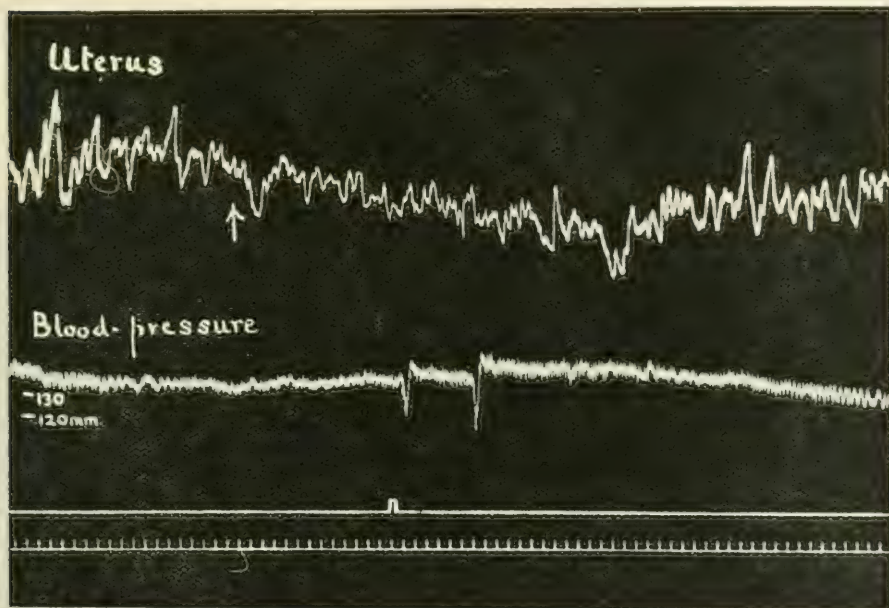


FIG. 8A

Cat, 3 kilos. Early pregnancy. Urethane. Abdomen opened in saline bath at 37°. Record of uterine contractions and carotid blood-pressure. Contraction of the uterus pulls down the lever, the movements being magnified twofold.

Effects of injecting 100 mgms. of clavin, dissolved in water, intravenously. No perceptible alteration in uterine tone or rhythm. The arrow indicates the time of injection for the tracing from the uterus.

For analysis the substance was recrystallised once with animal charcoal, and finally once without animal charcoal. It melted in a sealed capillary tube at 265°. Heated in an open tube, it yielded a crystalline sublimate, but also underwent a slight decomposition, and gave an odour very similar to that which leucin gives when heated in the same manner. The substance consisted of minute feathery needles, but sometimes rhomb-shaped plates were observed. With copper acetate it yielded a blue copper salt, slightly soluble in water. The general behaviour of the substance left no doubt as



to its identity with clavin. This identity was further supported by analyses, which, at the same time however, showed that the substance was not a chemical individual.

I. 0.1381 gramme gave 0.2630 gramme  $\text{CO}_2$  and 0.1168 gramme  $\text{H}_2\text{O}$ ; C = 51.94 per cent., H = 9.40 per cent.

II. 0.1607 gramme gave 0.3072 gramme  $\text{CO}_2$  and 0.1350 gramme  $\text{H}_2\text{O}$ ; C = 52.14 per cent., H = 9.33 per cent.

III. 0.1792 gramme gave 15.7 c.c. N at  $7^\circ$  and 770 mm.; N = 10.8 per cent.

IV. 0.1593 gramme gave 14.4 c.c. N at  $10^\circ$  and 765 mm.; N = 10.9 per cent.

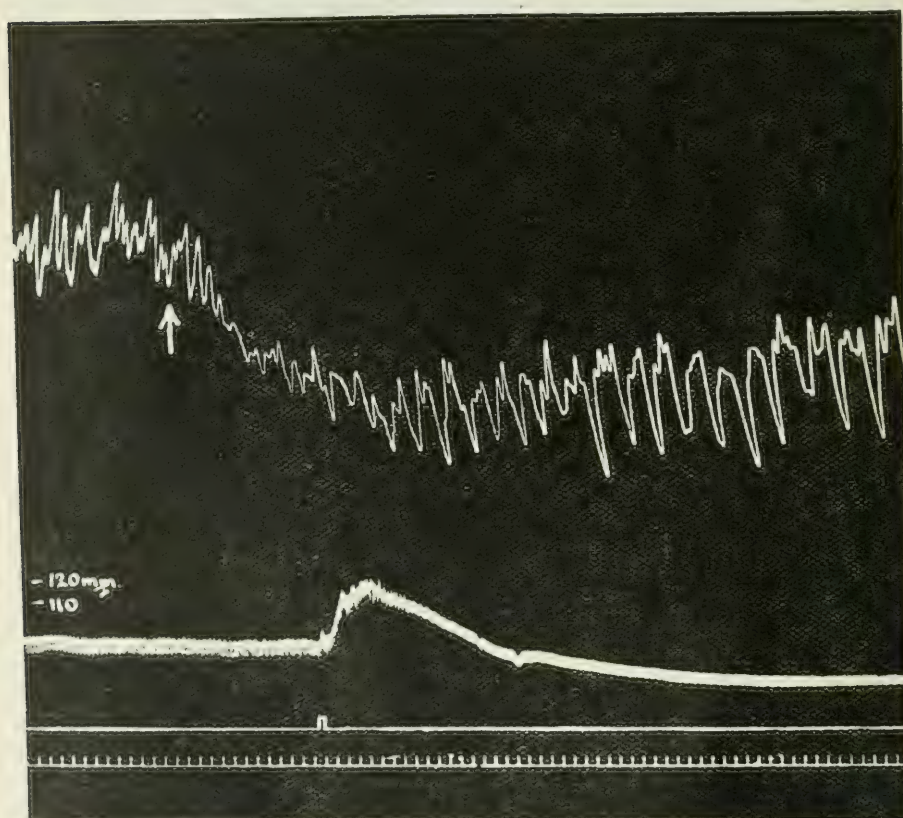


FIG. 8B

*Same animal as in FIG. 8A*

Effect of a later injection of 2 mgms. ergotoxine phosphate dissolved in water. The condition of the animal being poor, through long exposure of the viscera to saline, there is no great rise of blood-pressure, and only a moderate effect on the uterus. The tone and rhythm are, however, distinctly augmented.



Molecular weight determinations gave the following results:—

I. 0.215 gramme in 14.46 grammes water gave  $\Delta = 0.24^\circ$ ; M.W. = 117.

II. 0.100 gramme in 14.76 grammes glacial acetic acid gave  $\Delta = 0.030^\circ$ ; M.W. = 876. 0.230 gramme in 14.76 grammes glacial acetic acid gave  $\Delta = 0.093$ ; M.W. = 650.

III. By a vapour pressure method due to one of us,<sup>1</sup> in aqueous solution, 0.1049 gramme in 3.9877 grammes water was intermediate between urea solutions of 0.18 and 0.22 gramme molecules per litre; M.W. 118 to 146, mean 132.

IV. By the same method in glacial acetic acid solution 0.138 gramme in 5 grammes of glacial acetic acid was intermediate between benzil solutions of 0.14 and 0.18 gramme molecules per litre; M.W. 150 to 194, mean 172.

As the analytical results were not in agreement with a single formula of the magnitude indicated by the molecular weight determinations, the substance was converted into its copper salt, by boiling with saturated copper acetate solution. As did Vahlen, we succeeded in separating the copper compound into two fractions differing as regards their solubility in water. Regenerating the substance from the less soluble copper salt, which was by far the larger fraction, we found it to have a composition but slightly different from that of the original mixture (C = 52.6 per cent., H = 9.7 per cent.). Since the properties of the substance suggested that it was leucin in an impure form, and since, as is well known, it is very difficult to purify amino-acids by recrystallisation, we adopted Fischer's process of separation by distillation of the esters.<sup>2</sup> The presence of leucin in ergot was suspected long ago by Buchheim (10).

5.5 grammes of clavin, prepared by ourselves, was suspended in 50 c.c. of absolute alcohol, and saturated with dry hydrochloric acid gas. The substance soon dissolved completely; the solution was then evaporated *in vacuo* at a temperature below  $60^\circ$  to remove water formed during the reaction; a light brown syrup remained behind, which on cooling formed a mass of crystals of the hydrochloride of the ester. This was redissolved in absolute alcohol, again saturated, treated with potassium carbonate and caustic soda, and extracted with ether in the manner described by Fischer. During

1. *Journ. Chem. Soc.*, LXXXV, p. 286, 1904.

2. *Ber. deutsch. chem. Gesellsch.*, XXXIV, p. 433, 1901.

this process a small quantity of an amino-acid was apparently reformed and became suspended in the ether in fine crystals, which were filtered off.

On fractionating the mixture of esters at 9 mm. distillation began at  $79^{\circ}$ , and 3.4 c.c. distilled over below  $85^{\circ}$ .

The thermometer then rose rapidly, remaining for a short time at  $126^{\circ}$  to  $127^{\circ}$ ; a few drops of a somewhat viscid and slightly coloured distillate were collected between  $120^{\circ}$  and  $130^{\circ}$ . On further heating, the residue in the flask apparently boiled at a still higher temperature, but it was too minute to be distilled over and underwent decomposition. The boiling-point of the lower fraction corresponded very closely to that given by Fischer for leucin ester. The boiling-point of the higher fraction in conjunction with its solubility in water (which excludes phenyl-alanin) indicated that it consisted chiefly of aspartic ester, possibly with traces of glutaminic ester.

For identification the leucin fraction was hydrolysed by boiling with twenty times its volume of water for five hours; plates were obtained apparently identical in all respects with a specimen of leucin prepared from casein.

On analysis—

0.1239 gramme gave 0.2494 gramme  $\text{CO}_2$  and 0.1110 gramme  $\text{H}_2\text{O}$ . Found: C = 54.68 per cent., H = 9.95 per cent. Calculated for  $\text{C}_6\text{H}_{13}\text{O}_2\text{N}$ : C = 54.96 per cent., H = 9.92 per cent.

The copper salt was wholly insoluble in methyl alcohol, thus indicating the absence of iso-leucin.<sup>1</sup> A measurement of the optical rotation in 20 per cent. hydrochloric acid gave the following results:—

$$\alpha_D = +0.79^{\circ}; \quad c = 5\%; \quad l = 1 \text{ dm}; \quad [\alpha]_D = +15.8^{\circ}$$

The value given by Schulze for *l*-leucin under these conditions is

$$[\alpha]_D = +17.5^{\circ}.$$

The amount of the fraction collected between  $120^{\circ}$  and  $130^{\circ}$  was too small to admit of an attempt at hydrolysis, so the impure ester was analysed as such.

1. Ehrlich, *Ber. deutsch. chem. Gesellsch.* XXXVII, p. 1809, 1904.

0.1125 gramme gave 0.2060 gramme  $\text{CO}_2$  and 0.0870 gramme  $\text{H}_2\text{O}$ .  $\text{C} = 49.9$  per cent.,  $\text{H} = 8.6$  per cent.; calculated for aspartic ester,  $\text{C}_8\text{H}_{13}\text{O}_4\text{N}$ :  $\text{C} = 50.8$  per cent.,  $\text{H} = 8.0$  per cent.

We thus proved that the specimen of clavin prepared by ourselves consisted almost completely of leucin and aspartic acid. The analytical results of the original mixture, given above, indicate that its approximate composition was six parts of leucin to one part of aspartic acid. Such a mixture would have  $\text{C} = 52.3$  per cent.,  $\text{H} = 9.3$  per cent.,  $\text{N} = 10.7$  per cent.; the mean of the values found was:  $\text{C} = 52.1$  per cent.,  $\text{H} = 9.4$  per cent.,  $\text{N} = 10.8$  per cent.

We next examined two commercial specimens of clavin by the same method. The first of these contained 40 per cent. of di-hydrogen potassium phosphate. On esterification 1.93 grammes left undissolved 0.4 gramme of potassium chloride (equivalent to 0.73 gramme or 38 per cent. of di-hydrogen potassium phosphate). On distillation the same fractions were obtained in the same relative proportions as in the case of the specimen prepared by ourselves.

The leucin, obtained by hydrolysis, was analysed:—

0.1231 gramme gave 0.2468  $\text{CO}_2$  and 0.1061 gramme  $\text{H}_2\text{O}$ .  $\text{C} = 54.68$  per cent.;  $\text{H} = 9.58$  per cent.  $\text{C}_5\text{H}_{13}\text{O}_2\text{N}$  requires:  $\text{C} = 54.96$  per cent.;  $\text{H} = 9.92$  per cent.

The second commercial specimen yielded only 1 per cent. of ash. 4 grammes gave 3.2 grammes of leucin ester and 0.3 gramme of a higher boiling fraction. This specimen contained 52.6 per cent. of carbon and 10.3 per cent. of hydrogen.

The values given above for the molecular weight of 'clavin' in aqueous solution (117 and 132; Vahlen found on the average 122) are in agreement with those of leucin (131) and aspartic acid (133). In acetic acid solution, however, we obtained a higher value (172), and the mean of Vahlen's determinations in this solvent was 247.5. It was on this higher value that he founded his formula  $\text{C}_{11}\text{H}_{22}\text{O}_4\text{N}_2$ . We have found, however, that in glacial acetic acid solution the molecular weight of pure leucin (from casein) is abnormal.

0.084 gramme in 14.74 grammes acetic acid gave  $\Delta = 0.044^\circ$ ; M.W. = 506.

0.210 gramme in 14.74 grammes acetic acid gave  $\Delta = 0.168^\circ$ ; M.W. = 332.

No significance can, therefore, be attached to the molecular weight determinations of 'clavin' in acetic acid.

The other properties of 'clavin,' described by Vahlen, are consistent with the fact that it is chiefly composed of leucin and aspartic acid. The separation into two copper salts a more soluble (that of aspartic acid) and a less soluble (that of leucin) is thus rendered intelligible. The percentage of chlorine in the hydrochloride of leucin is 21.2 per cent., and in that of aspartic acid 20.0 per cent.; Vahlen found for the mixture 22.1 per cent.

The result of our chemical investigation of clavin completely explains our failure, and that of Cushny, to obtain any effect on the uterus with this substance.

#### SUMMARY AND CONCLUSION

1. Of the physiological effects described as characteristic of ergot, the alkaloid ergotoxine produces in very small dosage:—

(a) The effects ascribed by Kobert to sphacelinic acid, and by Jacoby to sphacelotoxin, viz.:—Ataxia, dyspnoea, salivation, gastro-intestinal irritation, and gangrene.

(b) The stimulant effect on plain-muscular organs—in particular the arteries and the uterus—and the subsequent selective sympathetic motor paralysis given by many ergot preparations (Dale).

2. Ergotoxine,  $C_{35}H_{41}O_6N_5$ , is the hydrate of the crystalline alkaloid ergotinine (Tanret),  $C_{35}H_{39}O_5N_5$ . Ergotoxine is itself amorphous, but yields crystalline salts, and further differs from ergotinine in being very soluble in alcohol. Its most important difference, however, is its intense physiological activity, ergotinine being but slightly, if at all, active when pure. Either alkaloid can readily be transformed into the other by chemical means.

3. The action of the pharmacopoeial extracts appears too great to be accounted for by the small amount of ergotoxine which they contain, and it seems likely that some other active principle is present in them.



4. This other hypothetical principle is not the 'clavin' of Vahlen, which is a mixture of leucin and aspartic acid, and is pharmacologically inert.

It will, perhaps, be useful to express in tabular form our conception of the relation to the ergot alkaloids, ergotinine and its hydrate ergotoxine, of the various other 'active principles' obtained from ergot, based on our own experiments and on those of other observers.

Ecboline and ergotine (Wenzell) = Mixtures of alkaloids, containing choline (Meulenhoff).

Amorphous ergotinine (Tanret) = Impure mixture of ergotinine and ergotoxine.

Picrosclerotine (Dragendorff) = Ergotinine, possibly mixed with ergotoxine.

Sclerocrystalline (Podwyssotski) = Ergotinine.

Sphacelinic Acid (Kobert) = Inactive resin with adherent alkaloid.

Cornutine (Kobert) = An alkaloidal resin, probably containing some ergotoxine, and also some other active substance, which may be a decomposition product of ergotoxine.

Cornutine (Keller) = Impure mixture of ergotinine with ergotoxine.

Chrysotoxin (Jacobj) = Inactive yellow colouring matter with a small proportion of adherent alkaloid.

Secalintoxin (Jacobj) = Mixture of ergotinine and ergotoxine.

Sphacelotoxin (Jacobj) = Impure ergotoxine.

Hydroergotin (Kraft) = Synonym for ergotoxine.

It will be clear from the above table that Tanret's conception of the nature of the active constituent of ergot approximated much more closely to that held by ourselves and by Kraft than did those of the intervening observers. The only modification necessary of the view put forward by Tanret, albeit an important one, was the recognition that his so-called 'amorphous ergotinine' was not merely physically different from the crystalline, but was largely composed of a separate though closely-related chemical individual, and that to this amorphous alkaloid, and not to its crystalline anhydride, were due the more prominent physiological effects of ergot. Led astray by Tanret's identification of the two alkaloids, and finding that the crystalline, and, therefore, pure, ergotinine was almost inert, other observers such as Kobert and Jacobj were induced to search in other

directions for a principle which Tanret had already so nearly isolated. Now that the nature of this principle is clear, a considerable simplification both of nomenclature and conception should be possible in the hitherto bewildering pharmacology of ergot.

We have pleasure in expressing our indebtedness to Messrs. F. H. Carr and W. C. Reynolds, whose assistance in preparing extracts on a large scale alone made our work possible; also to Mr. A. J. Ewins for valuable assistance in the details of the chemical investigation.

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*Note added May 8th, 1907.*—We have recently been in private communication with Prof. Vahlen, who is kind enough to inform us that he has been continuing his chemical examination of Clavin, and agrees that one of the constituents may be an amino-caproic acid. With regard to the other constituent, yielding the more soluble copper salt, he has departed from his original view that it was also a nitrogenous acid, and now holds that it is not an acid, and that it is the active substance.

ON THE TREATMENT OF TRYPANOSOMIASIS BY ATOXYL  
(AN ORGANIC ARSENICAL COMPOUND), FOLLOWED  
BY A MERCURIC SALT (MERCURIC CHLORIDE) BEING  
A BIO-CHEMICAL STUDY OF THE REACTION OF A  
PARASITIC PROTOZOON TO DIFFERENT CHEMICAL  
REAGENTS AT DIFFERENT STAGES OF ITS LIFE-  
HISTORY

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The experiments recorded below are of interest, first, from their special application to the prevention of the recurrence of trypanosomiasis after the parasites have been attacked and driven out of the blood by a remedial agent; and, secondly, from the more general point of view as an illustration of the important fact that when dealing with a parasitic protozoon which is attacked by a chemical substance or drug at one definite period only of its existence, it may happen that the drug may cause the parasite to pass into an encysted or a passive form which is no longer attacked by the drug and which, when conditions in the host have changed, may give rise to a fresh development of the parasite in its active form and a consequent recurrence of the disease. Further, the experiments show that a second substance used subsequently as a therapeutic agent may attack the passive form of the parasite and destroy it although this second agent may have no effect whatever upon the first or active form of the parasite and used alone as a curative agent would be entirely without effect.

1. A preliminary notice on this subject by us appeared in the *Annals of Tropical Medicine and Parasitology*, Vol. I, No. 1, p. 161, March, 1907.



It is already well known that in certain diseases due to protozoa a drug attacks the parasite at one period of its life cycle and is powerless at other times, as, for example, in the action of quinine upon the parasite of malaria.

Empirically discovered instances are also known where one drug is efficient at an early stage of a disease, and another drug is specific at a later stage, although our knowledge is not yet sufficiently advanced to enable us to state whether or not this reaction of the disease-producing parasite to different drugs at different stages corresponds definitely to different stages of development of the parasite at these respective periods.

Thus in the case of syphilis, which has recently been shown to be caused by a protozoon, mercury is specific in its action in the earlier stages while iodides are without effect ; at a later stage, while mercury is still indicated, used alone it is insufficient, and the iodides show a well marked specific action.

It was reasoning on such lines which induced us to attempt by empirical experimentation to discover some drug which should prevent the recurrence of trypanosomiasis after the trypanosomes had been driven out of the blood by some drug, such as atoxyl, which possesses a specific action upon the usual active form in which the trypanosome is found in the blood and tissues.

The results hitherto obtained by experimenting with mercuric chloride in rats experimentally infected with nagana (*T. brucei*) from which the trypanosomes had been driven out of the blood by treatment with atoxyl, have been sufficiently encouraging to induce us to publish them, as an example of this principle of experimenting with a view to finding reagents capable of attacking protozoa at different stages. Our findings may perhaps encourage experimentation along similar lines in the case of other protozoa.

Various substances have been described by different investigators which possess the power in varying degree of driving trypanosomes out of the blood of animals infected with different forms of trypanosomiasis.

These substances when they have been demonstrated to have an

action upon one species of trypanosome have usually been shown later to affect the other well-known species of trypanosome also, although quantitatively the effect varies somewhat from species to species. Accordingly, with certain quantitative restrictions, trypanosomiasis may be regarded as a whole, and from the therapeutic point of view, one species may be taken as a sample in the first instance, although afterwards naturally the results so obtained must be extended by experimentation to the other important species.

As a material for experimentation, the *Trypanosoma brucei* presents many advantages, in the ease with which infection can be passed from animal to animal; the rapidity with which development occurs in the usual laboratory animals; and the comparatively short interval in which in the vast majority of cases the parasites recur again after treatment with the initial drug has been given and interrupted. On the other hand, the long latency of infection and development, and also after initial treatment the slow and irregular recurrence of the parasite, which may not take place for months or years, render the trypanosome of sleeping sickness (*T. gambiense*) tedious and uncertain for such work of testing the effect of a second drug.

Accordingly, we have used for our experiments chiefly a strain of *T. brucei*, but are now engaged in extending our observations to the trypanosome of sleeping sickness. Also in order to be able to carry out a large number of experiments we have used small animals (rats) but are now proceeding to experiment with guinea pigs, rabbits, dogs, and asses.

The remedies which have up to the present been shown to possess the effect of, at least initially or temporarily, driving trypanosomes out of the blood, may be divided into two classes, viz., (a) certain aniline colouring matters which possess a similarity in molecular constitution—of these the most effectual are trypanroth (Ehrlich and Shiga) (1) and p. di-amido-di-phenyl-urea + Ac.H [Ph.], p. di-chloro-benzidine + Ac.H. [Cl] (Mesnil, Nicolle, and Aubert) (2),<sup>1</sup>

1. These two colouring materials are supplied by the firm, Farbenfabriken vorm. Bayer & Co., Elberfeld, under the names, Afridol violet, and Afridol blue. It is a pleasure to express our indebtedness to Messrs. Bayer & Co. for their kindness in presenting us with quantities of these and other colouring matters for use in our researches.

and malachite green (Wendelstadt) (3) ; and (b) compounds of arsenic of which the most effectual by far is the organic aniline derivative known as atoxyl.<sup>1</sup>

We have, up to the present, used only atoxyl as the initial drug for driving out the parasite from the blood, but are at present investigating the effect of using the aniline colour preparations mentioned above for this purpose.

The beneficial action of arsenical compounds has been known for many years, having been first pointed out by the explorer Livingstone in regard to nagana. Since then many observers (Bruce, Lingard, Laveran and Mesnil, Thomas and Breinl, and others) have recorded the marked action of various forms of arsenical compounds administered in different ways, on the parasites in the blood stream of animals infected with trypanosomes of several different species.

It has been universally recognised, however, that in the vast majority of cases the protective effect of the usual forms of arsenic is only temporary and that, in the end, the parasites reappear in spite of steadily increased doses of arsenic until a point is reached at which the animal dies either of the disease or through poisoning by the arsenic.

A great advance was made on the treatment by ordinary forms of arsenic by Thomas alone, and in collaboration with Breinl (4). These authors introduced treatment by atoxyl, an organic compound of aniline and arsenic. This discovery was due to a search amongst the organic compounds of arsenic for one which would still retain its deleterious effect on trypanosomes but would be less toxic to the animal treated. A number of preparations were tried without result until atoxyl was found to possess both of the desired qualities to a marked degree. A large series of experiments on animals infected by various species of trypanosomes was carried out at the Run-corn Laboratory. It was found that the trypanosomes could be rapidly driven out of the blood by atoxyl, and that this substance was much more potent as a remedy than any other therapeutic agent hitherto

1. Sold by Lanolinfabrik Martinikenfelde, Berlin.



described, and it may here be added than any since described. As a result of their experiments, Thomas and Breinl recommended strongly the systematic treatment of sleeping sickness in man by atoxyl, and this has since been done by many observers (Kopke, Thiroux and d'Anfreville, Martin, van Campenhout, Koch and others) (5).

The results in delaying the rate of advance of the disease in the treated cases has been most gratifying, and treatment by atoxyl has now become a standard with all workers upon the subject, either alone or in conjunction with other remedies.

In one most important respect, however, progress has hitherto been lacking, and that lies in preventing a recurrence of the disease either on discontinuing the atoxyl treatment or, indeed, in preventing in a good many cases recurrence of the trypanosomes, even when dosing with atoxyl has been continued throughout the progress of the case.

Although there is some contradiction in the results obtained by different observers it may be said that the results on the whole obtained by treating sleeping sickness in man with atoxyl are very similar to those obtained by Thomas and Breinl in the treatment of animals experimentally infected with *T. gambiense*.

Just as in the case of animals, there appears to be a small percentage of cases in which after efficient treatment with atoxyl lapse does not take place, but in the great majority of cases which have been under observation for a sufficiently long period to form a judgment, recurrence takes place. After each recurrence the parasites can be driven out for a time with atoxyl but with increasing difficulty, and finally death occurs directly from trypanosomiasis.

In considering the permanent cure of the trypanosomiasis of *T. gambiense* in man, the tardy action of this parasite above alluded to must be borne in mind, and many of the cases recently reported as cured by atoxyl alone require longer watching before any definite conclusion can be drawn from them.

There are on the one hand undoubted cases where, in spite of continued treatment, relapses have kept on recurring, ending in the death of the patient, and on the other exceptional cases where after



much shorter treatment the trypanosomes have not reappeared and the cases as far as can be judged must be regarded as cured.

Similar exceptional cases due to some idiosyncrasies either in the animal or in the infection occur in every laboratory research, even on more rapidly fatal forms of trypanosomiasis such as nagana. Also in such cases even after months of waiting recurrence may sometimes take place. So that caution is necessary in all cases and safe conclusions can only be drawn by observing two conditions, viz., first, sufficiently long periods of treatment and watching; secondly, by the employment of the statistical method and avoiding judging from too few cases, or the behaviour of the exceptional cases.

To sum up the present position of our knowledge of the treatment of trypanosomiasis by atoxyl or the other remedies mentioned above. These substances are capable of temporarily causing the trypanosomes to disappear from the blood, and in a small percentage of cases reappearance is long delayed or may never occur; but in the vast majority of cases even when the parasites have been so caused to disappear for some time, reappearance takes place when treatment is stopped, or eventually, in many cases, even when treatment is continued throughout. The explanation of the apparent cures in sleeping sickness may be due chiefly to the more tardy appearance of the parasite characteristic of that affection, but even allowing all these cases there are recorded cases of persistent treatment with atoxyl for months in which the parasites continued in the blood and eventually produced death.

Several observers have used the different other trypanosome remedies in conjunction with the use of atoxyl with the object of avoiding the undesirable effects of prolonged use of arsenic in considerable quantities, and also to prevent the parasites becoming inured to the arsenic by holding them in check alternately with other non-arsenical drugs, such as the aniline compounds above mentioned.

Also, van Campenhout has used strychnine in conjunction with atoxyl, and a system of cold baths, in order to stimulate the depressed central nervous system. From this combination van Campenhout records very satisfactory results, although he does not claim that the

strychnine has any direct effect upon the parasites but that it supports and stimulates the central nervous system.

The combination or alternation of the aniline colours with atoxyl mentioned above does not appear to give much better results than atoxyl alone, as far as can be judged from the records. In all such cases two drugs have been used in combination or alternation which both act upon the ordinary active stage of the trypanosome. It does not appear to have occurred to previous observers to search for a drug which might be entirely inert upon this stage of the parasite, but might act upon the latent form of the trypanosome which must in all probability exist in blood or tissues while the active form is being held in abeyance by atoxyl or other drug (7).

This formed the starting point of our research, and we began our work by testing the effect of salts of other heavy metals than arsenic after the trypanosomes had first been driven out as completely as possible by one, or more, subcutaneous injections of sufficient doses of atoxyl.

A series of white rats were taken and inoculated as equally as possible with nagana, they were then observed daily until trypanosomes had appeared in large numbers in the blood of all members of the series; all members were then treated with atoxyl and the observations of the blood continued until all no longer showed trypanosomes in the blood. This stage was attained usually as the result of one injection of atoxyl but in most cases a second injection of atoxyl was given as a routine procedure in order to ensure complete disappearance of parasites in the usual or active form. When this position had been reached, the rats being used in the experiment were divided into two lots one of which received no further treatment and served as a control, while the other lot received injections three or four times of a solution of a salt of the particular heavy metal being tested after which treatment of this lot of animals also ceased. Observation was then kept up in all for reappearance of parasites in the blood, and the time of death of each animal was noted in cases where death occurred.

Working in this manner, salts of copper, lead and silver were tested without any positive results being obtained, but on testing

mercury in the form of mercuric chloride, a distinct and important positive result was fortunately obtained, the trypanosomes not reappearing in the majority of cases. A large percentage of the treated animals survived and are at present alive, several months now, in some cases, after full infection with a strong strain of nagana, while all the controls to all the experiments are now dead. In the case of two animals only from all our series was a recurrence of trypanosomes observed in spite of treatment with atoxyl followed by mercuric chloride.

In addition to the experiments on the action of mercury after atoxyl, we have carried out a series of experiments on the nature and chemical properties of atoxyl, with a view to a more rational knowledge of its mode of action and decomposition in the body, and the reason for its different action from certain other organic arsenic compounds such as the cacodyl compounds on the one hand and from the inorganic arsenic compounds on the other.

The experiments on treatment are given in Section A, those on the chemistry of atoxyl in Section B.

## SECTION A

### *The Treatment of Experimental Nagana in White Rats by Atoxyl, followed by a Mercuric Salt*

Both drugs were administered subcutaneously, in sterile aqueous solution, the atoxyl in 5 per cent. solution, the mercury salt as the liquor hydrargyri perchloridi B.P. containing 0.1 per cent. of mercuric chloride, or in one experiment as Donovan's solution (1 per cent. iodide of mercury and 1 per cent. of iodide of arsenic in water).

All the rats were inoculated subcutaneously with approximately 0.4 c.c. of blood taken from an infected rat on the second day of infection and mixed with sodium citrate solution.

The strains of *T. brucei* employed killed untreated rats in from five to seven days after inoculation, but in some of the later experiments a particularly virulent strain was used which killed rats in from two to three days.

This virulent strain was obtained from a rat infected by the ordinary strain used, in which trypanosomes had reappeared after treatment by atoxyl alone. This increase in virulence after treatment by atoxyl was more than once observed during our experiments, and it appears to us that this may assume a certain importance in regard to the treatment of trypanosomiasis by atoxyl alone, especially in the treatment of sleeping sickness.

The treatment by atoxyl was only commenced after the disease was well advanced, as shown by the presence of abundance of trypanosomes in the animal's blood.

It is of high importance that the solution of atoxyl should be prepared fresh immediately before use, as the substance undergoes rather rapid alteration, probably a hydrolytic change, on keeping in aqueous solution especially in the light. As a result one obtains with a kept solution more of the toxic action of inorganic arsenical preparations and less of the specific effects of the atoxyl. Both these effects are inimical to the obtaining of good results, the toxic action of the arsenic on the rat being increased and the destructive action on the trypanosomes being decreased. This change in an exaggerated degree is seen on injecting into rats very old atoxyl solutions, thus a solution which had been kept for seven months in the light caused, in amount which is safe with the fresh solution (0.5 c.c.), *death within four hours*, while a solution which had undergone somewhat less decomposition, having been kept for the same period in an amber-coloured bottle in a cupboard, caused death in 25 to 37 hours, when also given in 0.5 c.c. dose. Unfortunately this change in the atoxyl on keeping in aqueous solution was not known to us in the earlier part of our work, and we attribute some of the deaths occurring soon after treatment was begun to the toxic effect of inorganic arsenic from partially changed atoxyl.

Accordingly the precaution of only using freshly-made solutions may be recommended to those working with atoxyl in trypanosomiasis (6).

In regard to the mercury treatment, this must only be commenced after the trypanosomes in their usual well-known active form have been completely driven out of the blood.



It has been conclusively shown by the work of previous observers, and we have also taken the precaution to demonstrate by preliminary experiments of our own that mercury treatment *alone* will not affect the usual form of trypanosome in the blood.

It is after the trypanosomes have been driven out of the blood, or changed into some inert passive form that the mercury salts become effective. Hence atoxyl, or probably some other drug capable of causing the primary change must be used first, and *after* that the mercury salt.

It is also essential in order that good results may be obtained that the largest possible therapeutic dose of both the atoxyl and mercury salt should be employed.

The routine examination of the blood was made in fresh  $\frac{3}{4}$ -inch square coverslip preparations. Occasionally the blood of important animals was centrifugalised.

For purposes of comparison of the control rats, treated by atoxyl alone, with those treated by atoxyl followed by a mercuric salt, it may be stated here that, as a general rule, the *T. brucei* reappeared in the circulation of the rats treated by atoxyl alone in from sixteen to twenty-five days after the atoxyl was stopped.

Administration of atoxyl was commenced on the third to fifth day after inoculation, dependent on the time at which the parasites appeared in abundance in the blood. Subsequent treatment with the mercuric salt commenced on the eleventh day, dependent as above stated on the complete disappearance of the parasites from the blood.

For the first ten days or a fortnight after the completion of treatment the animal's blood was examined daily. As the animals lived longer the examinations became less and less frequent until they were done approximately weekly. The blood of any animal evidently ill was immediately examined and, if necessary, sub-inoculations were made.

## RECORDS OF EXPERIMENTS

*Experiment I.*—Four rats received 0.5 c.c. of a 5 per cent. solution of atoxyl on the eighth day after inoculation, the third day after trypanosomes had been observed in the blood. On the four following days 2 c.c. of the mercuric perchloride was given. *Trypanosomes were never afterwards seen in any of these four rats.* One died forty-two days after inoculation, cause of death not discoverable, but not accompanied by trypanosomes or showing symptoms of trypanosomiasis. A second, being moribund, was killed ninety-five days after inoculation. Trypanosomes were not seen at the autopsy on either animal, and a rat sub-infected from the rat killed has never shown trypanosomes. The remaining two rats of this experiment are still alive 181 days after inoculation. Rats and mice sub-inoculated from them on the 92nd and 156th days have never shown trypanosomes and are still alive.

*Experiment II.*—Six rats received 0.8 c.c. of the atoxyl solution in two doses on the second and third days after inoculation. The infection was severe; one rat died before, two others just after, the first dose of atoxyl. Two of the remaining three animals received no further treatment. One of these two died of trypanosomiasis in twenty-two days, the other in twenty-seven days after the cessation of treatment. In the sixth rat, the course of atoxyl was followed by 1.5 c.c. of mercury perchloride solution given in two doses on the fifth and eleventh days after inoculation. Trypanosomes never reappeared in this animal. Thirty-one days after inoculation the animal seemed ill; it was therefore killed and another rat was sub-inoculated. Trypanosomes never appeared in the sub-inoculated animal, which died of skin disease three months later.

*Experiment III.*—Five rats each received 0.5 c.c. of atoxyl solution on the third day after inoculation. Two of these rats received no further treatment; one died, cause of death not discoverable, sixteen days after inoculation, the other of trypanosomiasis in twenty-eight days, the parasites reappearing three days before death. In the remaining three rats the atoxyl was followed by 2.7 c.c. of mercury perchloride given in four doses on the fifth, seventh, tenth and eleventh days after inoculation. One died, no trypanosomes, in forty days, the remaining two are still alive 155 days after inoculation. Mice sub-inoculated from them 130 days after inoculation have not become infected.

*Experiment IV.*—Two rats each received 0.5 c.c. of atoxyl solution on the fifth day after inoculation. One had no further treatment and died three days later. The other received 1.9 c.c. of the mercury perchloride solution in three doses on the eighth, tenth and twelfth days after inoculation. *Trypanosomes reappeared on the fifteenth day.* Another 0.5 c.c. of the atoxyl solution was given at once but the animal died next day.

Treatment was probably commenced too late here; both animals were almost moribund when the atoxyl was given. It is possible that the result might have been better in the mercury-treated rat had a larger dose of atoxyl been given. One dose is probably insufficient for so heavily infected an animal. It is questionable whether the parasites were ever entirely absent from the circulation.

*Experiment V.*—Six rats were inoculated; one died on the third day. On the fourth day the remaining five rats received 1 c.c. of the atoxyl solution in two doses. Two had no further treatment. Trypanosomes reappeared in these two rats in twenty and twenty-three days respectively after the cessation of atoxyl, and they died two days later of trypanosomiasis (thirty days after inoculation). The remaining three rats, after the atoxyl treatment, each received 2.7 c.c. of the mercury perchloride solution in four doses on the fifth to eighth days after inoculation. Trypanosomes were not again seen in any of the three. One died (spleen enlarged but direct cause of death unknown) twenty days after inoculation; the other two are still alive 123 days after inoculation. Mice inoculated from them ninety-eight days after inoculation were not infected and are still alive.

*Experiment VI.*—Twelve rats were taken for experiment, four died during the night of the second day after inoculation. On the third and fourth days the remaining eight received 1 c.c. of the atoxyl solution in two doses. Four of these eight rats received no further treatment; one died almost immediately; parasites reappeared in the other three in from ten to forty-one days after the cessation of treatment, and all died in from sixteen to forty-nine days after inoculation.

On the fourth, eighth, tenth and eleventh days after inoculation 2.7 c.c. of mercury perchloride was given in four doses to each of the other four rats. One died after the first dose, all of the three others were negative until thirty-six days after inoculation when trypanosomes reappeared in one of them. The other two are still negative at eighty-four days after inoculation; mice sub-inoculated from them at fifty-nine days after inoculation have not shown trypanosomes.

The rat in which trypanosomes reappeared received 1 c.c. of the atoxyl solution in two doses on the day its relapse was detected, and the second day after. It then received 1.7 c.c. of mercury perchloride in three doses on the fifth, seventh, and ninth days after the relapse. Parasites again reappeared in fourteen days after the relapse. Atoxyl was again immediately given, 1 c.c. in two doses on successive days; the trypanosomes disappeared as usual from the peripheral circulation. The animal died unexpectedly seven days later. Trypanosomes had not reappeared in its peripheral blood; spleen and lymphatic glands were enlarged.

This is the only instance of a recurrence of trypanosomes after a satisfactory combined treatment by atoxyl and mercury. In this experiment treatment was certainly commenced very late. There was a smaller interval between the administration of the two drugs than is usual; but there were probably no infective trypanosomes left in the peripheral circulation after the atoxyl treatment since a rat sub-inoculated at that time has not since shown trypanosomes and still lives.

*Experiment VII.*—On the third and fourth days after inoculation ten rats received 1 c.c. of the atoxyl solution in two doses; two rats died during the night of the third day. Four of the remaining eight received no further treatment. Trypanosomes reappeared

in them in from thirteen to twenty-four days after the cessation of treatment, and they died two to three days later.

The other four rats received 2·7 c.c. of mercury perchloride solution in four doses on the sixth to tenth days after inoculation; trypanosomes were not again seen in any of them. One died, cause unknown, twenty-five days after inoculation. The remaining three are still alive fifty-six days after inoculation, and mice sub-inoculated from them on the thirty-first day are alive and have not shown trypanosomes.

*Experiment VIII.—Atoxyl followed by Donovan's solution.* Eight rats received 1 c.c. of a 3 per cent. solution of atoxyl in two doses on the fourth and fifth days after inoculation. Two died during the fourth night. On the sixth, eighth, and tenth days 1·5 c.c. of Donovan's solution was given in three doses to the remaining six rats. All of them are still alive forty-four days after inoculation; mice sub-inoculated on the nineteenth day are alive and have not shown trypanosomes.

### SUMMARY OF EXPERIMENTS

The following tabular statement presents in a summarised form the main results of the experiments :—

Total number of rats used for experiments	...	...	53
Number of deaths before any treatment, or at commencement of atoxyl treatment	...	...	14
I. <i>Effect of treatment by atoxyl alone, the treatment being stopped when blood was free from trypanosomes, i.e., after one or two doses :—</i>			
(a) Number of rats treated	...	...	14
(b) Deaths from trypanosomiasis	...	...	12
(c) Deaths from unobserved or unknown causes	...	...	2
(d) Percentage of survivals in rats treated by atoxyl alone <sup>1</sup>	...	...	0
II. <i>Effect of treatment by mercury salt, after exactly the same atoxyl treatment as in rats in I :—</i>			
(a) Number of rats treated	...	...	25
(b) Deaths from trypanosomiasis	...	...	2
(c) Deaths from unobserved or unknown causes	...	...	4

1. Including other work not described in the text, we have treated in all 113 rats experimentally infected with *T. brucei* with a single dose of atoxyl alone; of these only three have survived.



(d) Rats killed when unhealthy or moribund but showing no trypanosomes, and giving no trypanosomiasis on sub-inoculation	...	...	2
(e) Number of survivals	...	...	17
(f) Percentage of survivals	...	...	68

Particular attention may be drawn to the contrast between survivals after atoxyl alone, and atoxyl followed by mercury. The somewhat high percentage of deaths before treatment was begun or just at the commencement of atoxyl administration may be ascribed to two causes, the first and most important of these being that we purposely only worked with animals showing high infection, and therefore began treatment at a very late stage, and secondly, in earlier experiments the point as to freshness of atoxyl solution was not sufficiently appreciated, and in some instances there was evidence that these deaths at an early stage were due to intoxication by the deteriorated solution in animals already enfeebled by the trypanosome infection.

In concluding this section we may make some suggestions as to the mode in which the mercury prevents recurrence of the trypanosomes. One possible view would be that atoxyl usually kills all the trypanosomes save some more resistant forms.<sup>1</sup> The parasites are left in a 'weakened state' in which they are destroyed by the ordinarily inert mercury. Such an hypothesis indicates the successive combined employment of various trypanocidal substances in the treatment of trypanosome infections.

It may be that the parasite weakened but becoming immune, accustomed to the first substance administered may be killed by the unaccustomed second or third substance given immediately afterwards. Thus atoxyl might on this view be alternated with trypanoth or the aniline colours Ph and Cl, as suggested by Mesnil and Nicolle.

The objection to this view, however, is that as already stated above mercury salts alone without primary driving out by atoxyl (or

1. This suggestion was originally made to us by Dr. Thomas.

possibly by other primary trypanocides) have no apparent effect, and some amount of amelioration would certainly be expected did the mercury ions attack trypanosomes of ordinary form enfeebled by atoxyl.

Further, there is no evidence that weakening by atoxyl would lead to attack by mercury. Again we have evidence from the results of infection from rats in which trypanosomes have recurred after atoxyl treatment that the strain is now considerably more virulent than at first, and hence the trypanosomes appear stronger instead of weaker.

Finally, the combined and alternate using of atoxyl and other primary trypanocides such as the aniline dyes (Ph and Cl), in the hands of Mesnil and Nicolle, have not given appreciably better results as to cure or prevention of recurrence than the use of atoxyl alone. We are accordingly disposed at the present stage in our work, not to regard the mercury salts as being a parasiticide for the ordinary usual forms of trypanosome found active in the blood; but instead that another distinct stage or stages exists for which atoxyl does not act as a parasiticide or at least not as an effectual one, and that the mercury salt destroys this stage, and by so doing effects (after all the well-known stage has been first destroyed by atoxyl) the complete removal of the parasite from the organism and so prevents recurrence.

For clearness, let us call the usual well-known phase of the trypanosome A, and the other hypothetical phase, for the probable existence of which a certain amount of histological evidence already exists, B (7). Let us suppose that atoxyl is poisonous to A, but does not touch B; mercury salts, as we know from mercury treatment alone, do not touch A; let us suppose that they are, however, poisonous to B. Now if A and B are two phases in the life history of the parasite,<sup>1</sup> they may exist side by side in the blood and tissues, or one in the blood and the other in the tissues of an infected individual.

1. This does not of course exclude the existence of other phases in the tsetse-fly or other carrier of the parasite.

If now effectual treatment by atoxyl be given A is completely destroyed, but B is not affected.

Indeed, as is often found to happen when the environment is made inimical to the existence of any given phase of an organism, the act of destroying A may cause many of the parasites of that phase to give rise to individuals of phase B (7).

If now, all of A having been driven out, a parasiticide for B, such as the mercury salt of our experiments, be given, then both forms are destroyed, the individual is cleared of infection, and if both clearances be complete recurrence without re-infection becomes impossible.

If, on the other hand, the second parasiticide be not given, as long as the presence of the first parasiticide (atoxyl in this case) in the blood and body fluids of the infected individual keeps up detrimental conditions for A, passage from B phase back into A phase will be inhibited, and any few individuals of B phase passing back into A will be destroyed. When, however, from interruption of treatment, the pressure or concentration of the parasiticide (atoxyl) in the blood and lymph falls below a certain level then the parasites resting in phase B become once more free to pass back into phase A, and this takes place with recurrence of active infection. Further, even if the pressure of the first drug or parasiticide (atoxyl) be kept up continuously, the parasite in the resting phase B is always present unattacked by this drug, and all the time there is a tendency to escape, and there probably is a slow escape back into phase A, the escaping individuals being at first destroyed. We have here, however, all the conditions for gradual inurement to an inimical environment, and for the production of a strain of the organism immune to the parasiticide.

The result will be to produce an 'atoxyl-fast' organism which then resists an atoxyl treatment and persists in spite of continued manifestation of the drug.<sup>1</sup>

We do not desire to put this forward at the present time as more than a working hypothesis, but it at any rate possesses an interest as having led us to seek for a second remedy for the prevention of

1. Ehrlich has obtained such an 'atoxyl-fest' form of trypanosome (see note, p. 324).



recurrence of the parasite along new lines, that is to say, among substances which we, and others, had shown to possess no direct effect upon the usual form (A) of the parasite.

The previous attempts had been to alternate two substances both poisons for phase A; our scheme was to alternate a poison for phase A with some drug which should attack whatever A possibly passed into, or which was associated with A, viz., phase B.

We would venture to suggest similar experimental therapeutics in the case of other diseases caused by protozoa—the attempt in the case of chronic malaria, for example, to find some drug capable of preventing recurrences, which might possess not the slightest effect similar to quinine as a first drug.

Neither atoxyl nor mercury may prove of avail with other protozoa, but the fact that most protozoa possess widely different phases in their life cycle should be borne in mind and that a drug which is deadly in one phase may be quite inert and harmless in another, and accordingly the search is indicated for a suitable drug for each phase.

## SECTION B

### *Notes on the Chemical Composition and Chemical and Physical Properties of Atoxyl and on the Probable Mode of its Action*

The substance sold under this protected trade name by the firm Lanolinfabrik Martinikenfelde, Berlin, is a white crystalline powder readily and completely soluble in water, much less soluble but completely soluble in absolute alcohol.

The process for its preparation has not been published, but it is stated to be an anilide of metarsenious acid and its formula given as  $C_6H_5NH \cdot AsO_2$ .

We have been unable to obtain from the substance, however, any of the reactions which might be expected by analogy to occur with a body of such a constitution.

For example, it is not decomposed on warming with caustic alkalies as are most known anilides. It is not an ordinary salt of



aniline, as no aniline is set free on the addition of cold caustic alkali to its solution. Further, even on boiling with 40 per cent. caustic soda it does not distil off aniline readily as the well-known anilides all do. Long boiling with the strong alkali gives *traces* of aniline in the distillate as shown by the usual colour tests.

On dry distillation it gives a small yield of aniline.

It hydrolyses *slightly* more readily on long boiling in strongly acid solution. When boiled with fairly strong hydrochloric acid (about 2N was used) it gives a *slight* precipitate of  $As_2S_3$  on passing sulphuretted hydrogen through the boiling solution. But with more dilute acid, even on boiling, only a precipitate of sulphur is obtained.

This marked stability to alkalies and acids stands in marked contrast to its fairly rapid hydrolysis on standing at ordinary temperatures in aqueous solution, already alluded to in Section A.

Laveran (6) has shown that this hydrolysis proceeds much more rapidly on superheating in aqueous solution in an autoclave to  $120^{\circ}C$ .

These reactions indicate that the arsenic is much more stably held than if it were attached in a side chain as shown in the formula quoted above. The only known organic arsenical compounds showing a similar stability to the action of alkalies are those *containing the arsenic directly united to the benzene ring*, such as phenyl-arsenic acid and its salts  $[C_6H_5 \cdot As(OH)_2]$ , and  $C_6H_5 As(OH)(ONa)$ .

Both the physical properties of the aqueous solution and the analyses for arsenic and nitrogen which we have been able to make also throw doubts upon the formula assigned to it. Also, on incineration and ignition, a considerable amount of sodium carbonate is left behind, too large to be present as an impurity, and strongly indicating that the compound is a sodium salt of an organic arsenical compound.

#### *Physical Properties of the Aqueous Solution*

Two determinations of the freezing point in aqueous solutions of 5 per cent. and 3 per cent. gave values for  $\Delta$  of 0.606 and 0.367 respectively, leading to molecular weights of 153 and 151 respectively.

This low value for the molecular weight in aqueous solution points at once to electrolytic dissociation.<sup>1</sup>

On making determinations of the electrical conductivity we found this confirmed, the solution is a very good conductor, the 5 per cent. solution giving a resistance of 76 ohms, in a vessel in which  $\frac{N}{10}$  KCl gave a resistance of 53.5 ohms.

This proves the important fact in the consideration of how the atoxyl produces its therapeutic effects, that the substance is highly electrolytically dissociated, and that the activity is in all probability due to an ion which contains arsenic in organic combination, and not as might have been supposed to inorganic arsenic slowly set free from a very feebly dissociated compound, as the result of slow hydrolysis in the body.

The electrical conductivity is also opposed to the view of an anilide composition for the compound.

#### *Boiling Point of Alcoholic Solutions*

An attempt was made to obtain the molecular weight free from the disturbing influence of electrolytic dissociation by making determinations of the rise of boiling point of solutions in absolute alcohol. It was found, however, that in alcohol the substance is in colloidal solution, no measurable rise in boiling point being found even in saturated solution.

#### *Effects of Incineration*

One gramme of the atoxyl was taken and heated on platinum, at first over a Bunsen, and then to bright redness with the blowpipe flame. Afterwards it was twice moistened with strong hydrochloric acid, the residue extracted with distilled water and the amount of chloride determined.

On first heating with the Bunsen it commences to char without previously melting or volatilizing. As it chars it gives off arsenical fumes, and a cacodyl-like odour is obtained; after this first incineration

1. Taking the molecular weight as 239 (*vide infra*), the  $\Delta$  obtained above leads to an ionization of 58.2 per cent., assuming that ionization into two ions occurs.

the weight of residue was 0.37 gramme (weight of atoxyl taken, 1 gramme); on moistening with distilled water, and then heating to bright redness for some minutes over the Bunsen, the weight of residue was 0.274 gramme. At this stage a very brilliant sodium flame was obtained.

The residue was moistened with hydrochloric acid and a very brisk effervescence was obtained showing the presence of a carbonate of a fixed alkali. On reheating a splendid sodium flame was obtained, and more  $\text{As}_2\text{O}_3$  was given off. The heating after re-moistening with hydrochloric acid was repeated, and the residue was finally heated to bright redness in the blowpipe flame when it fused. The final weight of the residue was 0.224 gramme = 22.4 per cent. of the atoxyl taken originally.

These results appear to us to show unmistakably that atoxyl is a sodium salt. If the 22.4 per cent. of residue obtained above be taken as being sodium chloride this leads to 8.8 per cent. of sodium in the original atoxyl.<sup>1</sup>

#### *Determinations of Nitrogen*

The percentage of nitrogen was estimated in two Kjeldahl determinations and one by Dumas' method.

The two Kjeldahl determinations gave 4.7 and 4.9 per cent. of nitrogen respectively, and the Dumas gave 4.83 per cent. Taking 4.8 per cent. as an average, this gives a molecular weight of 292 for one nitrogen atom.

It may be pointed out that these nitrogen determinations which are concordant among themselves are quite different from the percentage required by the reputed formula representing atoxyl as an anilide.

1. In a subsequent experiment made expressly to determine exactly the percentage of sodium by incinerating in presence of excess of sulphuric acid to drive off all the arsenic, from 1 gram of atoxyl a weight of 0.2184 of dry  $\text{Na}_2\text{SO}_4$  was obtained giving 7.1 per cent. of sodium. The sulphate was then precipitated as  $\text{BaSO}_4$  and the percentage of sodium calculated from the weight of this precipitate gave 7.5 per cent. Taking 7.5 as the percentage of sodium, the molecular weight for one sodium molecule works out to 307.

*Determinations of Arsenic*

These were first attempted by heating with strong nitric acid in sealed tubes by Carius's method, but erroneous results were obtained probably due to the stability of the compound causing incomplete breaking up by the fuming nitric acid. Later the method of fusing with caustic alkali was employed, followed by determination of the arsenic as  $\text{Mg}_2\text{As}_2\text{O}_7$ . This method was suggested to us by the fact that the analogous phosphorus compound to what we suspected atoxyl to be, viz., amido-phosphenylic acid, breaks up readily on fusing with alkalis into aniline and phosphoric acid.<sup>1</sup>

In the analysis the weight of atoxyl taken was 0.6476 gramme, the weight of the  $\text{Mg}_2\text{As}_2\text{O}_7$  precipitate was 0.3987, yielding 29.65 per cent. of arsenic. This gives a molecular weight of 253 for one atom of arsenic.

*Analysis of the Silver Salt*

The percentage of silver in the silver salt produced by precipitation of the aqueous solution by silver nitrate solution, was also estimated by the usual method for organic silver salts, and gave as the mean of two determinations 35.83 per cent. leading to a molecular weight for one atom of 302 for the dry silver compound and of 217 for the dry sodium salt (atoxyl) or adding for the percentage of water (*vide infra*) = 271.

*Behaviour with Salts of Heavy Metals*

Atoxyl forms insoluble salts with the heavy metals such as silver and copper. On addition of a solution of silver nitrate to a solution of atoxyl in distilled water a heavy precipitate, pure white in colour, is obtained not soluble in excess of the silver nitrate but readily soluble in either ammonia or nitric acid. A solution of copper sulphate gives a canary-yellow precipitate, which is also soluble in either ammonia or nitric acid. The colour of the precipitate with silver nitrate shows the absence of either arsenious or arsenic acids. Phenyl-arsenic acid gives similar insoluble salts with silver and copper.

Since atoxyl gives aniline as a decomposition product on hydrolysis in aqueous solution, we are inclined to regard it, from this and the

1. It was found later that a rapid and accurate method of determining the arsenic consists in destroying by a mixture of sulphuric and nitric acid (10 c.c. of sulphuric and 5 c.c. of nitric to 1 gramme of substance) in a Kjeldahl flask, neutralizing with ammonia, precipitating with magnesia mixture, collecting on a Gooch filter, and weighing as  $\text{Mg}_2\text{As}_2\text{O}_7$ . Analysis by this method gave 26.2 per cent. = mol. wt. of 286.



above evidence, as in all probability a sodium salt of amido-phenyl-arsenic acid or some derivative of such an acid, containing the arsenic directly united to the benzene ring.

We are at present engaged in attempts to synthesize this and similar arsenical compounds, with a view to testing their efficiency as parasiticides for trypanosomes and other parasitic protozoa.

#### MODE OF THERAPEUTIC ACTION OF ATOXYL

It is of high scientific interest to consider how atoxyl produces its therapeutic action.

At first from its published formula as an anilide, we were inclined to think that this was very feebly ionized in solution so as to yield a low pressure or concentration of the arsenical ion, and that this pressure, without becoming at any time so high as to produce poisonous results, was maintained for a long time after the atoxyl had been administered by the large non-ionized portion of the anilide becoming slowly ionized as the arsenical ion of the ionized portion became used up by trypanosomes and tissues.

On this view, the difference in therapeutic action between inorganic arsenical compounds and an anilide such as atoxyl is described to be would have lain in the fact, that the concentration attainable with the inorganic compound being sharply limited by its toxic action, much inorganic arsenic cannot be given and the initial concentration very rapidly falls off; on the contrary in the feebly ionised organic anilide there would be a bank to draw upon in the large non-ionized fraction, and with such a dose as would give the same initial concentration in arsenical ion, the drop afterwards would be infinitely slower so that a sustained effect would be obtained without poisoning the animal treated. Also, the difference between one organic compound and another as between the ineffectual cacodylates and the atoxyl might have found an explanation in difference in dissociation, the cacodylates being so feebly dissociated as not to be capable of acting on the trypanosomes.

We must admit, however, that our work on the chemistry of atoxyl and its physical properties has caused us reluctantly to abandon

this simple explanation, and come to the conclusion that the action is a specific one due to a peculiarly constituted organic arsenic-containing ion.

The chemical considerations have shown that the fresh solution contains its arsenic very strongly attached and that aniline is not readily detached, and this view is supported by the physiological action of the drug when pushed; as has been shown by other observers. The poisoning effects with a large dose are neither those of arsenical poisoning nor of aniline poisoning. Nor with long continued use are the chronic effects of arsenic on the nervous system obtained, nor the anaemia, nor effects on blood corpuscles of a haemolytic agent such as aniline. The toxicity of atoxyl is only about  $\frac{1}{80}$ th of that of arsenic, and as just stated even then the symptoms are not those of acute arsenical poisoning; nor is there the action of free aniline, a much less amount of aniline than that contained in the atoxyl causes acute poisoning.

But these results cannot be explained on the basis of feeble dissociation, for as shown above by the electrical conductivity, atoxyl is very highly dissociated.

Hence it appears to us that the action is not to be ascribed either to an inorganic arsenical ion, or aniline, slowly formed by decomposition of the atoxyl, *but to direct and specific action of a complex organic ion containing both the aniline and arsenical groups.*

This view is in our opinion supported by the rapidity with which the atoxyl acts, a single dose given to a highly infected animal may cause entire absence of parasites from the blood on the following morning.

This exceedingly rapid action is hardly compatible with a slow decomposition of the drug.

#### CONCLUSIONS

I. Parasitic protozoa which show different phases in their life history may be attackable at one phase by a drug which is entirely inert and therapeutically useless at another phase, and conversely a drug which is without action on the first phase may be specific in its action upon the second phase.

2. For this reason, in the study of experimental therapeutics applied to protozoan parasites, not merely one drug must be tried, or an alternation of two drugs which have a lethal effect upon the parasite at one and the same stage only, but rather a drug which kills at one stage having been discovered; for the prevention of recurrence a second drug should be sought out which will attack the parasite in the succeeding stage, and this drug may not be found amongst others which kill at the first stage, but may well be sought outside that circle.

3. A special study has been submitted of atoxyl treatment followed by a mercury salt in rats infected by nagana (*T. brucei*), and it appears that such combined treatment gives better results than uncontinued treatment by atoxyl alone, although mercury salts alone have no action on the trypanosome.

4. It is suggested that the combined treatment should be given a careful trial in natural trypanosome infections of man and animals.

5. The treatment must be commenced as soon in the infection as possible; full therapeutic doses of both drugs must be given; fresh solution of atoxyl must be used; and the mercury salt begins to take effect when the parasites have been driven from the circulation by the atoxyl.

6. The substance atoxyl is in all probability not an anilide, but a sodium salt of an organic acid containing an amidogen group, and an arsenic radical directly united to a benzene ring.

The aqueous solution is strongly electrolytically dissociated, giving in consequence an apparently low molecular weight by the freezing point method, and possessing a high electrical conductivity.

Except on standing in aqueous solution, it is a most stable compound, and neither aniline nor arsenic are easily detachable from its molecule by chemical means.

Its toxic properties are neither those of arsenic nor of aniline even when pushed to excess, and its therapeutic action is rapid, from this and its high conductivity showing high dissociation, the conclusion is drawn that its activity must be ascribed not to free inorganic arsenical ions or to free aniline, but to a complex organic ion containing both the arsenical and aniline radicals.

Atoxyl forms insoluble salts with silver and copper, which are insoluble in neutral solution but soluble in ammonia and in nitric acid.

Our thanks are due to our colleagues Mr. E. S. Edie, Dr. W. Spence and Mr. J. E. Southcombe for much valuable advice and assistance especially in the chemical part of the work.

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*Note added May 6th, 1907.*—After our paper was concluded we received a reprint from Professor Ehrlich (*Chemo.-therapeutisch Trypanosomen-studien, Berl. klin. Wochensch.*, 1907, Nos. 9-12) in which he, in collaboration with Bertheim, has established that atoxyl is the sodium salt of para-amido-phenyl-arsenic acid, with four molecules of water of crystallization. Our analyses strongly support such a view except that we have found a somewhat lower amount of water.

A determination of the loss of weight on heating to a constant weight at 145°C. for some hours gave a loss of weight of 0.1984 grammes in 1.001 grammes of atoxyl, giving a percentage of 19.81 of water. This comes to slightly over three molecules of water of crystallization instead of four to the molecule of sodium-amido-phenyl-arsenate. The amount of water in commercial samples of atoxyl probably varies somewhat. Our analytical figures as well as the reactions and physical properties of atoxyl, as above stated, agree so closely with those which a salt of amido-phenyl-arsenic acid would show that we regard them as confirmatory of the view of Ehrlich and Bertheim.

The formula  $\text{NH}_2(\text{C}_6\text{H}_4)\text{AsO} \cdot \text{ONa} \cdot \text{OH}, 3\text{H}_2\text{O}$  requires  $\text{H}_2\text{O} = 18.43$ ,  $\text{As} = 25.6$ ,  $\text{N} = 4.77$ ,  $\text{Na} = 7.89$ , we have found  $\text{H}_2\text{O} = 19.81$ ,  $\text{As} = 26.2$ ,  $\text{N} = 4.8$ ,  $\text{Na} = 7.5$  per cent.



# ALLYL SULPHIDE: SOME ASPECTS OF ITS PHYSIOLOGICAL ACTION WITH AN ANALYSIS OF THE COMMON LEEK (*ALLIUM PORRUM*)

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## INTRODUCTION

The plants of this family are very widely distributed over the five continents, and distinct races of men in all quarters of the globe have used them largely as condiments without having any knowledge of their close relations. In Spain and Portugal onions are everyday articles of food, and onions, garlic and shallot are extensively used as foods and condiments in Russia, France, and all other European countries. The alliaceous diet of the peninsula was probably acquired from the Northern coast of Africa.

Over the whole of Northern Africa—from the Mediterranean to the Nile—garlic and the onion are highly esteemed as seasoners of the usual food. We are told that ‘Arab, Moorish, and Ethiopian tribes equally delight in them; and this taste is of very remote origin. The Israelites, during their sojourn in the wilderness, murmured, saying, “We remember the cucumbers and the melons, the leeks, the onions, and the garlic.”

‘Among the ancient Egyptians themselves the onion formed an object of worship: and the modern Egyptians assign it a place in their paradise. To the present day the onion of the Nile borders possesses a peculiar excellence and flavour.’<sup>1</sup>

Plants of this class have considerable nutritive value when used as food, but it is to the presence of a volatile oil that they owe their importance as condiments; this oil is allyl sulphide ( $\text{C}_3\text{H}_5)_2\text{S}$ , a yellow liquid possessing a characteristic odour of garlic. Com-

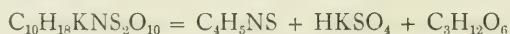
1. Johnson and Church, *Chemistry of Common Life*, p. 450.

mercially it is sold as oil of garlic, being prepared by the steam distillation of garlic. In this process 1 oz. of oil is obtained from 30 to 40 lbs. of garlic. Allyl sulphide occurs in variable but small amounts in the following plants:—The common onion (*Allium cepa*), cultivated garlic (*A. sativum*), leek (*A. porrum*), shallot (*A. ascalonicum*), common ramson (*A. ursinum*), chive (*A. schænoprasum*), rocambole (*A. scorodoprasum*), Indian onion (*A. leptophyllum*), *Thlaspi arvense alliaria officinalis* (*A. moly*).

The strength of the flavour depends mainly on the relative amount of oil present. Whether the universal selection of these plants as condiments is due to the physiological effect of the essential oil, or whether they have attracted attention through the psychological effect of their characteristic odour, it is impossible to say.

In the different varieties of mustard seed substances exist which are capable of forming another allyl derivative when under suitable conditions.

These two substances are potassium myronate and a ferment myrosin.<sup>1</sup> When a solution of myrosin or an extract of white mustard is added to an aqueous solution of potassium myronate the latter decomposes into mustard oil (allyl isothiocyanate), acid potassium sulphate and glucose.



Hence myronic acid and its salts are glucosides.

It is impossible to say whether in the alliaceous plants examined the allyl sulphide exists entirely preformed, or whether under appropriate conditions it arises from a glucoside precursor. At all events, the majority of it exists preformed, and if any is formed subsequently the amount is very slight.

On account of the close similarity of these plants, one was taken as a type of chemical composition, viz., the leek. This was used on account of the ease of obtaining it in a fresh condition.

For the investigation of the physiological effect, onions (the strongest procurable), garlic, and allyl sulphide itself were used.

1. *Jour. of Pharm.*, XCVI, 39.

## CHEMICAL ANALYSIS

(By C. LOVATT EVANS)

Johnson and Church<sup>1</sup> give as the composition of the onion :—  
 Water 91·0, flesh formers 1·5, starch<sup>2</sup> 4·8, fat 0·2, ash 0·5, other  
 substances 2·0 = 100.

Our results with the analysis of leeks according to Dragendorff's  
 method<sup>3</sup> were as follows :—

Water and volatile oil	...	...	...	...	...	88·00
Fixed oil (and glucoside ?)	...	...	...	...	...	0·65
Resinous substance	...	...	...	...	...	0·48
Organic acids, colours, etc., precipitable by lead acetate	...	...	...	...	...	1·10
Sugars (and extractives, etc., not precipitated by lead acetate)	...	...	...	...	...	2·24
Gums and pectous substances soluble in water	...	...	...	...	...	2·20
Proteins, etc.	...	...	...	...	...	1·55
Pectous substances soluble in dilute NaOH	...	...	...	...	...	1·69
Cellulose	...	...	...	...	...	0·77
Soluble in Br. NH <sub>4</sub> OH	...	...	...	...	...	0·46
Soluble Ash (= Cl, SO <sub>4</sub> and P <sub>2</sub> O <sub>5</sub> of K and Na)	...	...	...	...	...	0·72 (5% Cl)
Insoluble Ash <sup>4</sup>	...	...	...	...	...	0·14
						<hr/> 100·00

*Sugars and Gums* are the only constituents that call for special  
 mention.

A certain amount of a reducing sugar (dextrose) is present  
 normally in the plant, but on boiling with water the reducing power  
 of the solution is increased considerably. This is due to the fact  
 that a gummy substance is hydrolysed by the action of the hot water  
 and the acid juice, with the formation of a sugar.

The sugar so produced yields a crystalline osazone (M.P. =  
 196° C.).

The gum itself was found to be a reducing one, even after repeated  
 precipitation with alcohol and with hydrochloric acid and lead acetate.

1. *Ibid.*, p. 89.

2. This is really 'carbohydrate.'

3. Allen, *Commercial Organic Chemistry*, Vol. I, p. 429.

4. The composition of this ash is as follows :—SiO<sub>2</sub> 55·0, Fe<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> and Al<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 13·1, CaO 7·4,  
 CO<sub>2</sub> 1·25, SO<sub>3</sub> 2·3

It is readily extracted even from the fresh leek or onion by cold water, and it reduces, even when freed from sugar as above. The gum yields a slightly alkaline solution, which is precipitated as follows :—

*Readily precipitated* by basic lead acetate, lead acetate and ammonia, baryta water, and alcohol.

*Less readily precipitated* by lead acetate, lime water, dilute hydrochloric acid, sulphuric and phosphotungstic acids, acetic and tannic acids, acetic acid and potassium ferrocyanide, hydrochloric acid and potassium mercuric iodide, picric acid.

*It also yields the following reactions* :—Xanthoproteic—very slightly ; iodine—a pale brown colour ; strong mineral acids—brown on boiling.

The *Volatile Oil* was allyl sulphide ( $C_3H_5$ )<sub>2</sub>S. No trace of allyl isothiocyanate was found in the fresh onion or leek. The actual amount of allyl sulphide in even the strongest onions or in garlic is very small (not exceeding 0.1 to 0.2 per cent. in garlic). Traces of allyl oxide are not infrequently present.

#### PHYSIOLOGICAL PROPERTIES

1. *Absorption*.—The oil is absorbed unchanged, since experiments show that it is unaltered by prolonged gastric and pancreatic digestion. The absorption is a slow process, and often takes place with difficulty from the mucous membrane of the intestines and perhaps, to a certain extent, from the stomach also. It may also be absorbed through the skin, as in manipulating allyl sulphide in the laboratory, and after the application of a garlic poultice, in which cases its absorption in traces is indicated by its presence in the breath after a short time.

2. *Excretion*.—That the odour of allyl sulphide persists in the air expired is a well known fact, which shows that the lungs are a channel for its excretion, and also that the act of excretion is rather slow—a day or two being necessary to eliminate quite a small quantity. Its excretion in the milk excreted by cows eating alliaceous plants is a source of much annoyance to many dairy farmers.



Excretion by the skin is a common though not an invariable result of eating garlic or onions.

Lastly, it is never completely absorbed from the intestine, or, what is less likely, it is absorbed and re-excreted, so that it is almost constantly present in the faeces of persons eating alliaceous plants.

According to Johnson and Church<sup>1</sup> the odour of these plants is so persistent that it is said to be recognisable 'in the excreta of persons who have consumed the oil in the form of a sauce into which a chive or onion has been dipped.'

We have never been able to obtain evidence pointing to the excretion of allyl sulphide by the kidneys, though on many occasions it was searched for. For example, 50 grammes of strong English onion was eaten raw at 3.45 p.m., and the urine collected for seventeen hours afterwards. Volume, 575 c.c.; specific gravity, 1021; reaction, acid. Though the large amount taken caused the skin to smell of the sulphide, no traces of it could be discovered in the urine.

### PHYSIOLOGICAL EFFECTS

3. *General.*—The first effect of a moderate dose is an excitation of the central nervous system, followed by the usual compensation, viz., depression.

This we believe to be the most marked physiological property of allyl compounds in general, the extent of the excitement varying slightly with different compounds.

Thus allyl thiocyanate 'appears to exert a poisonous action, inasmuch as on working with the substance for some time headache, nausea, and nervous excitement are noticed.'<sup>2</sup> We have ourselves noted a similar effect with allyl iodide.

Allyl sulphide taken in the form of garlic, onions, or leeks, immediately before retiring at night may produce either an excited or depressed state of the nervous system, or both in succession, according to the dose.

1. *Ibid.*, p. 449.

2. Roscoe and Schoebemmer, Vol. III, Pt. ii, p. 389.

Upon persons of a neurotic tendency, garlic or onions, even in small quantities, cause extreme excitation, accompanied in some cases by vomiting, and in others by profuse salivation during sleep, followed by the extreme of depression. Allyl compounds and alliaceous plants all evidently owe their physiological effects to an action on the brain—chiefly upon the medullary centres in causing the physical effects, and on the cerebral regions in causing the psychical ones.

*Supposed Solvent Action for Urates.*—Allyl sulphide was found to exercise practically no solvent action upon either uric acid or urates. Whatever other therapeutic properties this compound may possess, therefore, it is certainly of no value in this relation.

#### SPECIAL PHYSIOLOGICAL EFFECTS AS TESTED ON LIVING ANIMALS

(By E. WACE CARLIER, M.D.)

The experiments were performed upon rabbits and one cat. The animals were first completely anaesthetised under a bell jar either with ether or with chloroform, and subsequently in all cases a tube was inserted into the trachea, through which the anaesthetic was administered in such a way that the anaesthesia could be regulated to a nicety, the animals thereby kept perfectly motionless, and artificial respiration easily maintained.

The operative procedures consisted in inserting cannulae into the carotid artery and jugular vein, or in merely inserting the point of the hypodermic needle into the latter and holding it in position with an artery clamp. The respiratory movements were recorded either by encircling the lower part of the thorax with a thin rubber band, to which a silk thread working over pulleys upon an elastically-supported lever was attached, or by Head's method.

The time was electrically recorded in seconds.

Owing to the difficulty of obtaining allyl sulphide from plants in sufficient quantity for these experiments, synthetically made allyl sulphide was purchased and used undiluted and in 1 per cent. and 5 per cent. solutions in normal saline. The solutions in all cases gave

rather anomalous results, due, no doubt, to the fact that the oil does not form good emulsions with salt solution; so that although the mixtures were administered with care, it was quite impossible to be certain of the exact amount of the drug present in each minim used. With the undiluted drug the case was very different, the results obtained being constant.

Allyl sulphide acts directly upon both the respiratory mechanism and the blood pressure, but only indirectly upon the heart, probably as a result of asphyxiation, though the inhibitory action of the vagi as tested by the inductorium is diminished by about one-half, but was never found to be entirely abolished.

*Effect on Respiration and Blood Pressure.*—The following experiment may be taken as a type:—

In a rabbit weighing 2685 grammes, 15 minims (0·89 c.c.) of a 1 per cent. solution in normal saline was injected into the jugular vein, and produced a slight fall in blood pressure amounting only to 6 mm. of mercury. The pressure began to fall  $4\frac{1}{2}$  seconds after injection, reached a minimum at the 42nd second, and thereafter rose steadily to a maximum of 16 mm. of mercury 598 seconds after the injection, crossing the normal line at the 127th second. This was followed by a fall to normal at the 778th second. This small dose, therefore, caused a disturbance of the blood pressure lasting nearly thirteen minutes.

The respiration was likewise affected by this amount, being reduced from 51 to 39 per minute during the fall of pressure, and continuing to diminish both in rate and amplitude despite the subsequent pressure rise. At the 100th second the respirations had fallen to 30 per minute, became irregular in amplitude, and continued thus to the 200th second, when they again became regular, though small, with a rate of 33 to the minute, and remained so to the 480th second, when they again became irregular for ten seconds, and began to increase in rate and amplitude. They regained their normal rate and amplitude at the 560th second. The respiratory disturbance, therefore, lasted some nine minutes—i.e., a shorter time than that of the blood pressure.



About a quarter of an hour later 1 minim of the pure allyl sulphide was injected, and was followed by an immediate fall of blood pressure of 47 mm. of mercury in  $25\frac{1}{4}$  seconds, the fall reaching to within 15 mm. of zero. This was soon succeeded by a gradual rise, which lasted until artificial respiration was resorted to; after which the pressure again fell somewhat.

The respiratory movements diminished in amplitude immediately after the drug was administered, and their record was reduced in 100 seconds to a mere sinuous line, and, therefore, the aeration of the blood rapidly became inefficient, by which the rise in blood pressure already noted was probably produced. Even these sinuosities in the tracing disappeared a little later, and, as the heart was beating strongly, artificial respiration was resorted to and maintained for about 20 minutes, after which the animal began to breathe normally though feebly. Three-quarters of an hour later the animal had quite recovered from the drug, and was killed by opening the carotid.

(The tracing of this experiment was taken on a fast moving drum, and is too long for publication, and, therefore, a similar one with slow motion has been given in Fig. 1.)

One minim of allyl sulphide was, therefore, a fatal dose for this animal, and, generally speaking, it was found that the intravenous injection of  $\frac{1}{2}$  minim (0.3 c.c.) per kilo. of body weight was always fatal unless artificial respiration was performed.

This drug, therefore, kills by asphyxia brought about by paralysis of the respiratory centre in the medulla.

Another interesting point in the action of this drug is that when an animal has fully recovered from a non-fatal dose a second dose of the same strength and amount has practically no effect either on the respiration or blood pressure, a considerably stronger dose being required to produce any effect at all, the first having caused a considerable amount of immunity.

*Effect of Large Doses on the Blood Pressure.*—The injection of 5 minims (0.3 c.c.) of pure allyl sulphide not only arrests the respiratory movements, but at once, or within 15 seconds, causes a fall of blood pressure to zero, yet the heart keeps on beating forcibly for a much



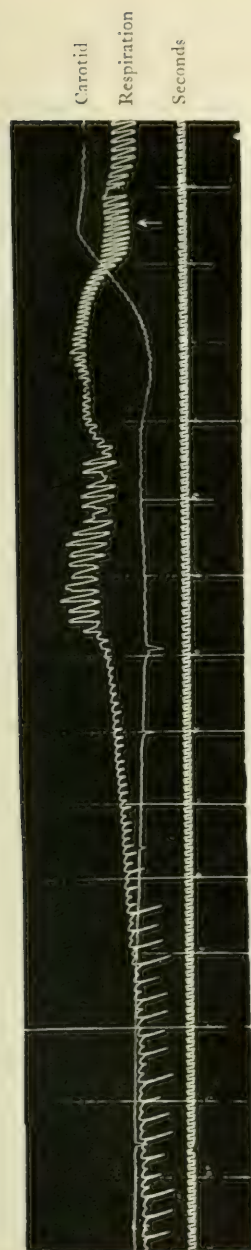


FIG. 1.—Effects upon the blood pressure and upon the respiration of the injection intravenously of 1 minim (0.06 c.c.) pure allyl sulphide in a medium-sized rabbit. This and all the other tracings are to be read from right to left. Scale of 2 : 5.



E D C B A

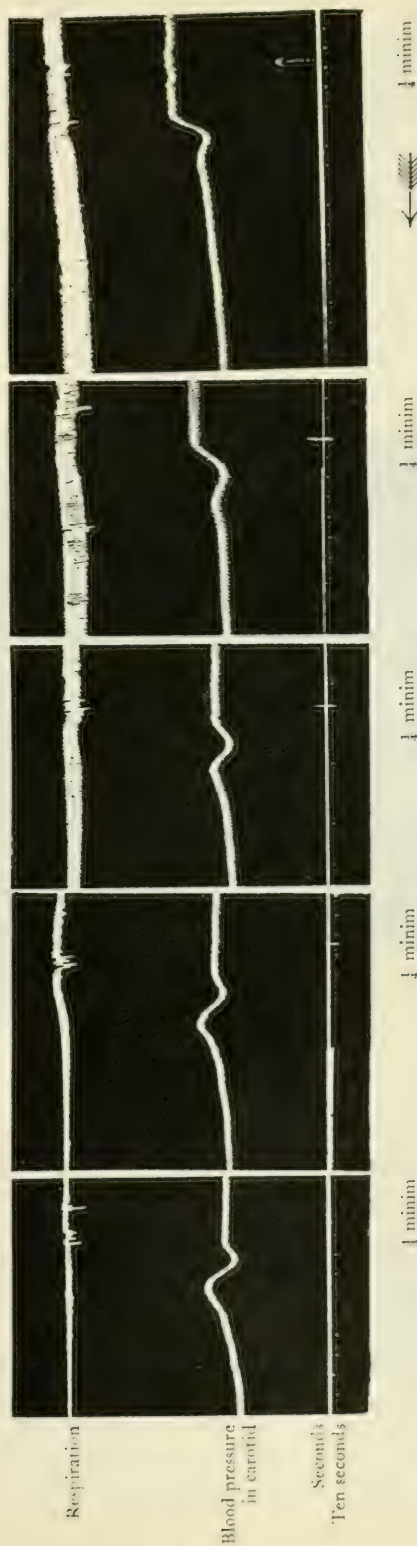


FIG. 3.—Effect of successive  $\frac{1}{4}$  minim (0.015 c.c.) doses of allyl sulphide injected into a rabbit weighing 2600 grams, upon the respiration and blood pressure. Scale of 2 : 5.

longer time, as may be seen from Fig. 2. No attempt was made to recover the animal from this dose with artificial respiration.

*Effect of Repeated Small Doses on the Blood Pressure.*—The effect of repeated small doses on the same animal is also interesting.

In a large rabbit  $\frac{1}{4}$  minim (0.015 c.c.) of the drug was injected as above, and produced on the blood pressure an immediate depressor effect, rapidly followed by a lesser pressor, which in turn is followed by a fall.

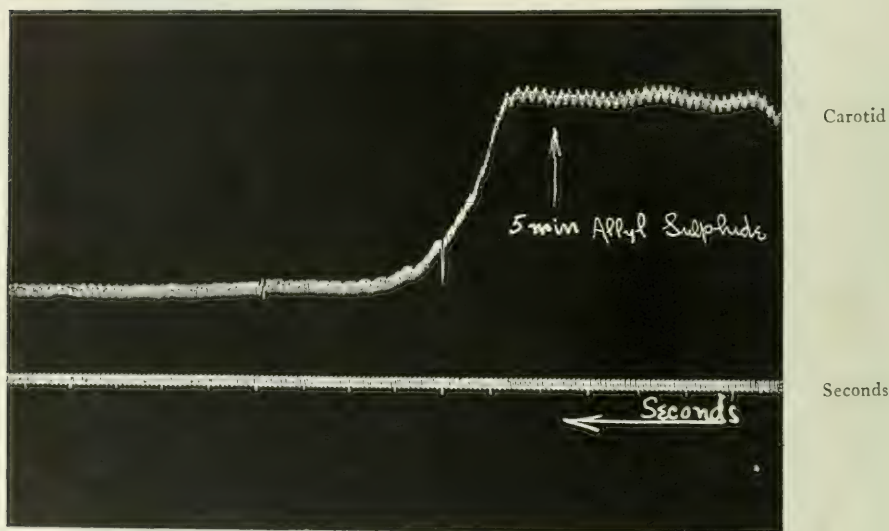


FIG. 2.—Carotid trace in a rabbit of 2300 grams showing the effect of a 5 minim (0.3 c.c.) dose of allyl sulphide upon the blood pressure. Scale of 2 : 3.

Shortly afterwards another  $\frac{1}{4}$  minim was injected, and followed as before by a depressor effect, though less rapidly produced, the subsequent pressor effect being more marked. Another  $\frac{1}{4}$  minim resulted in a less depressor effect followed by a pressor rise to slightly above the normal. Another  $\frac{1}{4}$  minim had a similar result with less depressor and still greater pressor after-effect, and another  $\frac{1}{4}$  minim increased the after pressor effect still more, but the depressor effect was never abolished.

The  $\frac{1}{4}$  minim doses were now given in rapid succession; the second being administered as the pressor effect of the first began to appear, and so with the third dose, with the result that a heaping up of the blood pressure to a considerable height was obtained. As shown by the tracing (Fig. 4) each pressor effect was checked, but a cumulative rise was obtained. At the top of this stair the heart began to fail, and the respirations quickly after died out, which resulted in death.

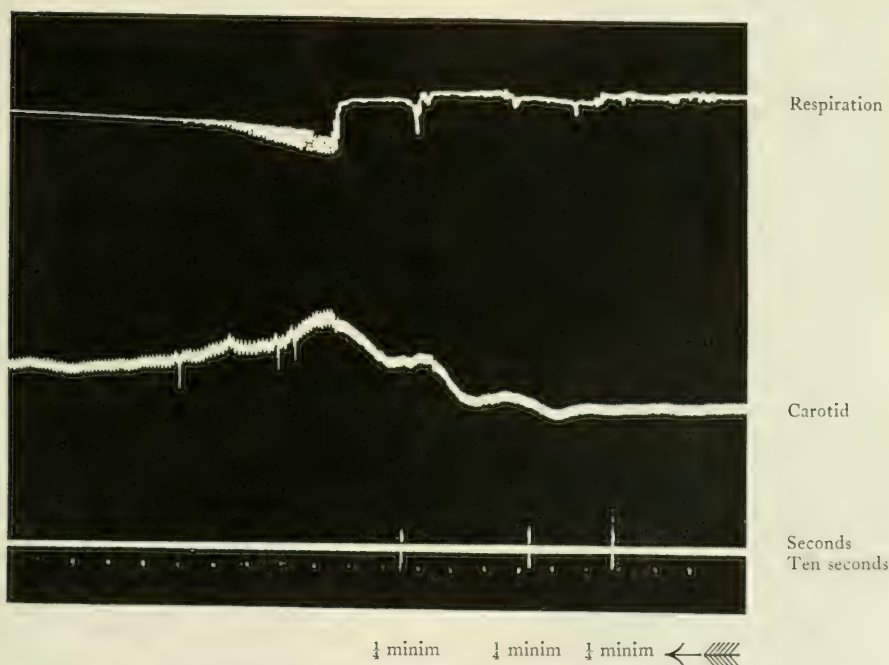


FIG. 4—Effect of three rapidly repeated doses of  $\frac{1}{4}$  minim (0.015 c.c.) allyl sulphide upon the same rabbit as in fig. 3, and subsequent to the injections there illustrated. The humped condition of the blood pressure trace is well shown.

Though the total amount of the drug given had amounted to  $2\frac{3}{4}$  minims (0.16 c.c.), the respirations had been little affected, gradually diminishing, but never ceasing until after the last dose had been administered. This immunisation seems, therefore, to apply to the respiratory rather than to the vaso-motor mechanism.

*Experiment to Determine the Seat of Action of the Drug upon the Blood Pressure Mechanism.*—To determine whether the vaso-motor centre was the seat of action of the drug a cat was chloroformed, pithed, and decerebrated; artificial respiration established, and the usual operations for a blood pressure experiment performed. Five minims (0.3 c.c.) of pure allyl sulphide were injected into the jugular vein, and produced somewhat tardily a slight depressor effect amounting only to 7 mm. of mercury. A further injection of 10 minims (0.6 c.c.) was then given, which caused a further fall of 3 mm. So that 15 minims (0.89 c.c.) of pure drug only resulted in a fall of 10 mm. mercury, which is less by 6 mm. mercury than that produced by a stimulation of the vagus before administration of the drug.

This is very different from the result obtained with even small doses of the drug on rabbits with intact medulla, presenting no comparison with the fall obtained by 5 minims (0.3) in the rabbit that yielded Fig. 2. No doubt the small depressor effect manifested was due to the action of the drug upon the supplementary vaso-motor centres present in the spinal cord, and we must conclude, therefore, that allyl sulphide acts on the vaso-motor centre and not on the musculature of the vessels, which was indeed to be expected from previous experiments with the drug on frogs' muscles, all of which gave negative results.

The vagus was again stimulated with the same strength of current as before, and produced arrest of the heart beat, and a stimulus with a current of double the strength gave the same result, but unaccompanied in both cases by any fall of blood pressure.

(The cat was used in this experiment because pithing in a rabbit results in a fall of blood pressure almost to zero owing to the paralysis of the great vessels of the abdominal cavity that supervenes, rendering it quite unsuitable for such experiments.)

*Effect of the Drug on the Heart and other Muscles.*—When allyl sulphide is dropped upon or injected into the myocardium of pithed frogs no alteration is produced thereby either in the rate, amplitude, or rhythm of the contractions, yet in a few cases large doses diminished the rate and increased the force of the contractions, and caused



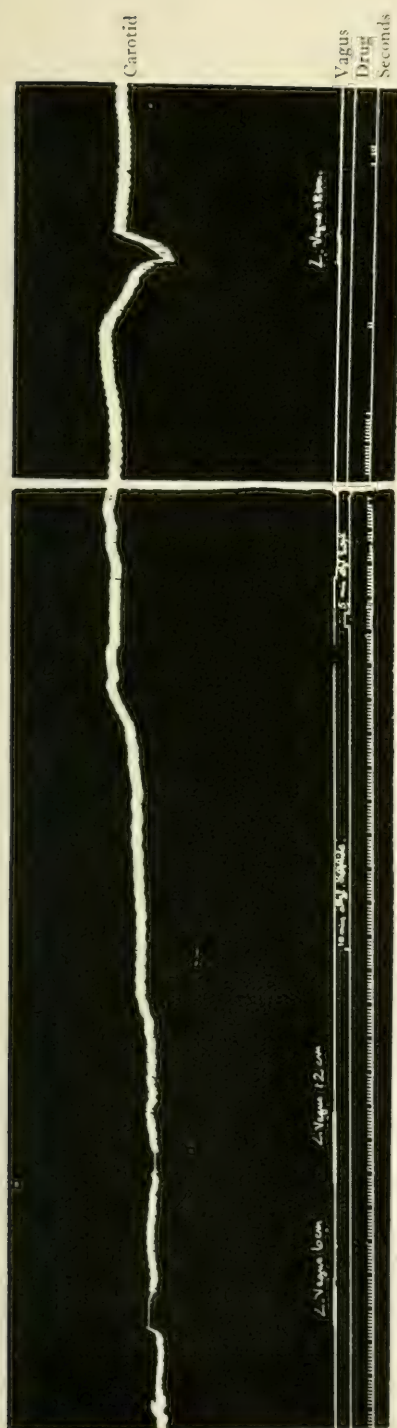


FIG. 5.—Cat weighing 2340 grams, shewing the effect of injection of 5 minims (0.3 c.c.) and then of 10 minims (0.6 c.c.) of pure allyl sulphide upon the blood pressure after destruction of the vaso-motor centre in the medulla, and also the effect of vagal stimulation upon the heart and blood pressure before A and after B the injection of allyl sulphide. Scale of 1 : 2.

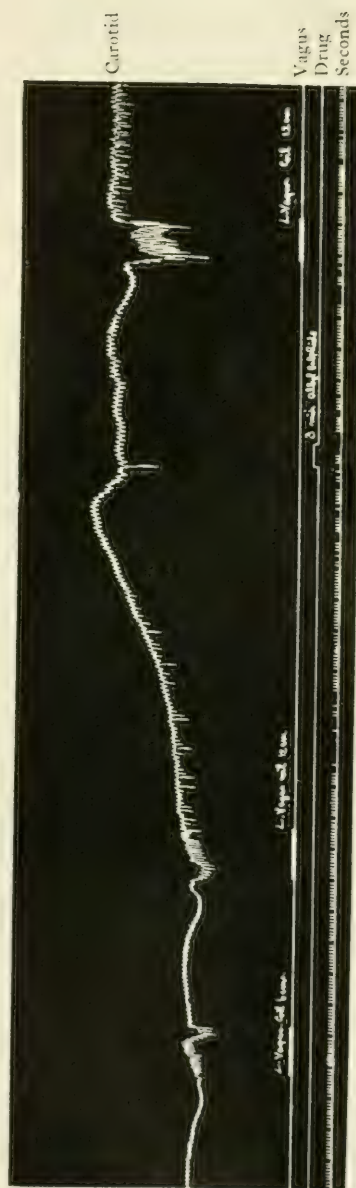


FIG. 6.—Carotid tracing in a rabbit weighing 2670 grams. The left vagus was stimulated before injection of three minims (0.18 c.c.) of allyl sulphide and again after the drug had produced a considerable fall in the blood pressure. Scale of 1 : 2.

some irregularity, even arresting the heart in diastole in one case; but this is quite exceptional, and due probably to the experiments having been done in winter. Mr. Cranston Walker, at my request, repeated these experiments upon a number of frogs, and in one case only was any change in the heart beat recorded.

With the mammalian heart in both rabbit and cat, injection into a vein of even fatal doses of the drug produced no direct effect upon it, as judged by the pressure tracings obtained from the carotid artery, except such as are normally present in death from asphyxia.

Only one experiment was performed in this relation upon a rabbit, in which the thorax was opened after the establishment of artificial respiration, and the heart so exposed made to record the beating of its auricles and ventricles over separate pulleys. In this case injection of the drug into a vein remained without effect, as did also direct injection of it into the myocardium.

In the case of the pithed cat the thorax was opened at the end of the experiment, and allyl sulphide dropped on the heart without effect, and even a considerable dose (a hypodermic syringe full) injected into the myocardium also failed to alter the beat in any way.

We may, therefore, safely conclude that the drug has no direct effect upon the heart.

Similarly, when injected into the skeletal muscle of the frog (gastrocnemius) no change is produced thereby in the force of the contractions, response to electrical stimuli, or the onset of fatigue, which is also borne out in the ergograms of one of us after large doses of leeks and onions eaten with the food as compared with those taken on other days on a regular diet.

*Effect on the Inhibitory Activity of the Vagus on the Heart as Ascertained by the Inductorium.*—In all cases in which the effect of vagal stimulation upon the heart beat and blood pressure was tried a marked decrease in vagal inhibition was demonstrated after injection of the drug into a vein in considerable doses, its depressor effect being reduced by one-quarter, or even, in some cases, by one-half,

that produced by the same strength of current before its administration, but whether the heart ganglion cells or the termination of the inhibitory fibres on the muscle are the seat of this phenomenon was not specially determined.

In the pithed cat the fall of blood pressure, well marked before the allyl sulphide had been administered, was not obtained upon stimulating the vagus after the drug had taken effect, though the heart was distinctly retarded and even arrested thereby.

### CONCLUSIONS

1. Allyl sulphide acts chiefly upon the respiratory centre in the medulla, paralysing it rapidly when large doses are intravenously administered.

2. The drug acts to a less degree upon the vaso-motor centre, diminishing its effect in the first instance, but followed, especially when small doses are given, by a subsequent rise of pressure that may persist for a considerable time.

3. When administered in small doses its effects upon the vaso-motor centre are more lasting than upon the respiratory, the contrary being the rule with large doses.

4. The drug diminishes to some extent the inhibitory action of the vagus upon the heart.

5. The drug has no direct effect upon the heart or upon skeletal muscle.

6. One-half minim of the pure drug per kilo. of body weight may be taken as a lethal dose, death resulting from asphyxia owing to paralysis of the respiratory centre, but if artificial respiration is had recourse to, recovery is complete within an hour.

7. After the effects of a non-lethal dose have passed off the animal is thereby immunised for a time against a similar and even a considerably larger dose.

8. Owing to its powerful action on the respiratory centre it cannot be recommended as a therapeutic agent for internal administration.

# ON THE EFFECT OF ACIDS, ALKALIS, AND NEUTRAL SALTS ON THE FERMENTATIVE ACTIVITY AND ON THE RATE OF MULTIPLICATION OF YEAST CELLS

BY ERIC DRABBLE, D.Sc., F.L.S., AND DAISY G. SCOTT, M.Sc.

(Received May 2nd, 1907)

In the present communication we give an account of some observations on the effect of acids, alkalis, and neutral salts on the rate of fermentation induced by yeast cells, and also some notes on the rate of multiplication of the yeast cells in culture media to which a neutral salt has been added to increase the osmotic pressure.

That plants grow less rapidly in concentrated than in dilute solutions was first shown by Jarius (1). His experiments were conducted upon the germination of seeds. Eschenhagen (2), working with Fungi, showed that the maximum strength in which the plants will grow differs from species to species, but for any one species is of the same osmotic strength for different salts.

Raciborski (3) stated that in *Basidiobolus* the maximal concentration for growth in solutions of sodium chloride is that exercising an osmotic pressure of about seventeen atmospheres, i.e., 6 per cent.

Livingston (4 and 5) finds that the Alga *Stigeoclonium* fails to produce zoospores in strong salt solutions.

## I. FERMENTATION IN THE PRESENCE OF ACIDS, ALKALIS, AND NEUTRAL SALTS

Solutions of sodium chloride, potassium chloride, sodium nitrate, potassium nitrate, hydrochloric acid, nitric acid, sodium hydroxide and potassium hydroxide were made up in strengths 0.1, 0.01, 0.001 and 0.0001 gram-molecular. One cubic centimetre of 50 per cent. solution of cane-sugar was made up to 26 c.c. with each of the solutions



of acid, alkali, and neutral salt named above. These were set up in duplicate in test tubes. To these were added yeast cells in such proportion that each tube, on a count being taken by means of the usual square-cell method, gave an average number of 26.5 cells per square. The dilution of the solution consequent on addition of sugar and yeast was calculated and allowed for, and the tubes were placed in the dark for a week. At the end of seven days the tubes were examined, and the state with regard to fermentation was noted. In the records given the note 'floating bubbles' represents a vigorous fermentation, while 'floating yeast' indicates the most active condition in this respect.

TABLE I

State of fermentative activity of Yeast in solutions of cane-sugar in various media after seven days.

Gram-molecular strength of medium				State of fermentative activity	
Sodium chloride	...	0.1	}	{ Fermentation, floating bubbles.	
" "	...	0.1	}	{ Fermentation, floating bubbles.	
" "	...	0.01	}	{ Much fermentation, floating yeast.	
" "	...	0.01	}	{ Fermentation, floating bubbles.	
" "	...	0.001	}	{ Much fermentation, floating yeast.	
" "	...	0.001	}	{ Much fermentation, floating yeast.	
" "	...	0.0001	}	{ Much fermentation, floating yeast.	
" "	...	0.0001	}	{ Fermentation, little floating yeast.	
Potassium chloride	...	0.1	}	{ Fermentation, floating yeast.	
" "	...	0.1	}	{ Little fermentation, little floating yeast.	
" "	...	0.01	}	{ Fermentation, little floating yeast.	
" "	...	0.01	}	{ Little fermentation, little floating yeast.	
" "	...	0.001	}	{ Fermentation, very little floating yeast.	
" "	...	0.001	}	{ Little fermentation, some floating bubbles.	
" "	...	0.0001	}	{ Fermentation, little floating yeast.	
" "	...	0.0001	}	{ Little fermentation, little floating yeast.	
Sodium nitrate	...	0.1	}	{ Fermentation, floating yeast.	
" "	...	0.1	}	{ Fermentation, floating yeast.	
" "	...	0.01	}	{ Fermentation, little floating yeast.	
" "	...	0.01	}	{ Fermentation, floating yeast.	
" "	...	0.001	}	{ Fermentation, little floating yeast.	
" "	...	0.001	}	{ Fermentation, floating yeast.	
" "	...	0.0001	}	{ Fermentation, floating yeast.	
" "	...	0.0001	}	{ Fermentation, floating yeast.	

Potassium nitrate ...	O'I	}	{ Some fermentation, floating yeast.
" " ...	O'I	}	{ Some fermentation, little floating yeast
" " ...	O'OI	}	{ Much fermentation, floating yeast.
" " ...	O'OI	}	{ Fermentation, floating yeast.
" " ...	O'OOI	}	{ Much fermentation, floating yeast.
" " ...	O'OOI	}	{ Fermentation, floating yeast.
" " ...	O'OOOI	}	{ Fermentation, floating yeast.
" " ...	O'OOOI	}	{ Fermentation, floating yeast.
Hydrochloric acid ...	O'I	}	{ No fermentation.
" " ...	O'I	}	{ No fermentation.
" " ...	O'OI	}	{ Very little fermentation, 3 floating bubbles.
" " ...	O'OI	}	{ Very little fermentation, few floating bubbles.
" " ...	O'OOI	}	{ Little fermentation, few floating bubbles.
" " ...	O'OOI	}	{ Some fermentation, floating bubbles.
" " ...	O'OOOI	}	{ Little fermentation, few floating bubbles.
" " ...	O'OOOI	}	{ Fermentation, floating bubbles.
Nitric acid ...	O'I	}	{ No fermentation.
" " ...	O'I	}	{ No fermentation.
" " ...	O'OI	}	{ Little fermentation, no floating bubbles.
" " ...	O'OI	}	{ Very little fermentation, no floating bubbles.
" " ...	O'OOI	}	{ Little fermentation, few floating bubbles.
" " ...	O'OOI	}	{ Some fermentation, floating bubbles.
" " ...	O'OOOI	}	{ Some fermentation, very little floating yeast.
" " ...	O'OOOI	}	{ Some fermentation, very little floating yeast.
Sodium hydroxide ...	O'I	}	{ No fermentation.
" " ...	O'I	}	{ No fermentation.
" " ...	O'OI	}	{ Some fermentation, trace of floating yeast.
" " ...	O'OI	}	{ Little fermentation, floating bubbles.
" " ...	O'OOI	}	{ Some fermentation, floating yeast.
" " ...	O'OOI	}	{ Some fermentation, floating bubbles.
" " ...	O'OOOI	}	{ Fermentation, floating yeast.
" " ...	O'OOOI	}	{ Fermentation, floating yeast.
Potassium hydroxide ...	O'I	}	{ No fermentation.
" " ...	O'I	}	{ No fermentation.
" " ...	O'OI	}	{ Some fermentation, floating yeast.
" " ...	O'OI	}	{ Little fermentation, floating bubbles.
" " ...	O'OOI	}	{ Fermentation, floating yeast.
" " ...	O'OOI	}	{ Fermentation, floating yeast
" " ...	O'OOOI	}	{ Fermentation, floating yeast.
" " ...	O'OOOI	}	{ Fermentation, floating yeast,

A consideration of the results summarised in the preceding table shows clearly that none of the neutral salts employed in the media exercises a marked inhibiting action on the fermentative activity of the enzyme within the range of strengths used. Both hydrochloric and nitric acids in concentration 0·1 gram-molecular completely inhibit the fermentative action; in 0·01 gram-molecular concentration the yeast is still slightly active, while in 0·001 and 0·0001 the fermentation is progressively greater.

In the case of sodium and potassium hydroxides the case is generally similar; in 0·1 fermentation is completely inhibited. In 0·01 it is only slight, but is more pronounced than in the same concentration of an acid. This is doubtless partly due to the fact that the evolved carbon dioxide is absorbed by the solution of hydroxide, whereby the amount of this substance in solution is reduced. In 0·001 and 0·0001 the fermentation is considerably more active.

## II. EFFECT OF THE SOLUTION ON THE YEAST CELLS

After seven days the state of the cells in the media containing hydrochloric acid, potassium hydroxide and potassium chloride was examined. When the cells were scattered and not budding the presumption is that they were inactive, a conclusion entirely supported by the state of fermentation in the tubes. When the cells instead of being uniformly scattered were found generally to be aggregated into clumps the activity was greater, while in the most active and healthy cells buds were being produced and active reproduction was going on.

A measurement of the mature cells in these tubes was made. The results of these observations are recorded in the following table :—

<i>Hydrochloric Acid</i>				
Strength in gram-molecules		Condition of the cells		Average diameter of the cells
0·1	...	Scattered, not clumped	...	7·3
0·01	...	Slightly clumped	...	9·4
0·001	...	Distinctly clumped	...	9·4
0·0001	...	Clumped and budding	...	9·4

<i>Potassium Hydroxide</i>			
Strength in gram-molecules	Condition of the cells		Average diameter of the cells
0·1	...	Scattered, not clumped	9·8
0·01	...	Slightly clumped, some buds	9·7
0·001	...	Much clumped and budding	9·7
0·0001	...	Much clumped and budding	9·7

<i>Potassium Chloride</i>			
0·1	...	Very much clumped, budding	9·5
0·01	...	Very much clumped, budding	9·8
0·001	...	Very much clumped, budding	9·9
0·0001	...	Very much clumped, budding	9·9

It will be noticed that in the hydrochloric acid tubes the cells are scattered, and, judging from their size, are killed at 0·1 gram-molecular strength. As has been shown elsewhere (6), yeast cells shrink when killed in acid. In the solutions of less strength the cells are clumped and are active, possessing a uniform average size in all the strengths. For potassium hydroxide the same holds true, only in 0·1 gram-molecular strength the cells are not reduced in size being, indeed, rather larger than in the weaker strengths.

In these lower strengths of alkali they possess a uniform average size rather greater than the size in the equivalent strengths of acid. In the neutral salt, potassium chloride, the cells are clumped and actively budding in each tube, but here it is interesting to note that the osmotic concentration of the solutions seems to come into play and results in progressively smaller-sized cells in 0·0001, 0·001, 0·01, and 0·1 gram-molecular solutions.

After seven days the yeast cells in the tubes were counted by means of the squared-cell method with the following results:—

The original number of cells per square was 26·5.

<i>Sodium Chloride</i>			<i>Potassium Chloride</i>		
0·1	gram-mol.	32·1	0·1	gram-mol.	35·8
0·01	..	37·3	0·01	..	35·7
0·001	..	45·7	0·001	..	33·7
0·0001	..	37·9	0·0001	..	36·2



*Sodium Nitrate*

0·1	gram-mol.	31·6
0·01	„	32·3
0·001	„	34·2
0·0001	„	32·7

*Potassium Nitrate*

0·1	gram-mol.	56·5
0·01	„	46·9
0·001	„	44·2
0·0001	„	36·5

*Hydrochloric Acid*

0·1	gram-mol.	24·5
0·01	„	37·2
0·001	„	42·3
0·0001	„	44·8

*Nitric Acid*

0·1	gram-mol.	22·1
0·01	„	30·4
0·001	„	34·0
0·0001	„	50·3

*Sodium Hydroxide*

0·1	gram-mol.	22·6
0·01	„	29·4
0·001	„	42·1
0·0001	„	46·9

*Potassium Hydroxide*

0·1	gram-mol.	25·5
0·01	„	26·6
0·001	„	33·2
0·0001	„	46·7

This count of the cells yields some interesting figures. The original average number per square was 26·5. In all strengths of neutral salts an increase in the number of cells is manifest, as could have been predicted safely from the fact that they are actively budding in these tubes. In the acids of strength 0·1 gram-molecular, on the other hand, not only has multiplication failed to take place but an actual reduction has been brought about by a certain proportion of the cells having been killed. These killed cells are seen as transparent structures which have lost entirely their granular appearance and were not counted in estimating the numbers. In the other strengths of acid there has been an increase in number.

In the tubes of hydroxides again there has been an actual reduction in number of cells in 0·1 gram-molecular strength. In all the other tubes there has been an increase in number except in 0·01 potassium hydroxide where the number is approximately equal to that at the commencement of the experiment.

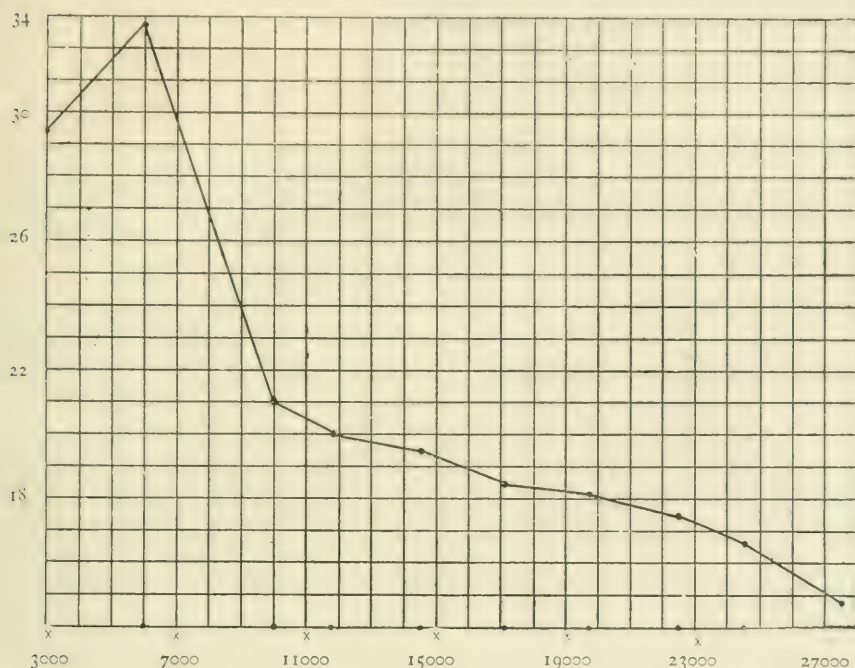
### III. MULTIPLICATION OF YEAST CELLS IN SOLUTIONS OF SODIUM NITRATE

A complete series of tubes was set up in duplicate in solutions of sodium nitrate from 0.1 to 1.0 gram-molecular strength. One cubic centimetre of 50 per cent. cane sugar solution was added to each tube, and yeast to produce eighteen cells to each square was placed in the solution, due allowance being made for the dilution thus caused.

The following results were obtained after seven days in the dark:—

Strength of solution in gram-molecules	Osmotic pressure of solution in millimetres of mercury	Number of cells	State of fermentative activity
0.1	3018	29.5	Fermentation, floating bubbles. Fermentation, floating bubbles.
0.2	6067	32.8	Fermentation, floating bubbles. Fermentation, floating bubbles.
0.3	8948	21.0	Fermentation, floating bubbles. Fermentation, floating bubbles.
0.4	11762	20.0	Fermentation, floating bubbles. Fermentation, floating bubbles.
0.5	11490	19.5	Fermentation, floating bubbles. Fermentation, few floating bubbles.
0.6	17287	18.5	Little fermentation, 1 bubble. Little fermentation, 2 bubbles.
0.7	19829	18.2	Little fermentation, 2 bubbles. Little fermentation, 3 bubbles.
0.8	22541	17.6	No active fermentation, no bubbles. No active fermentation, no bubbles.
0.9	24021	16.7	No active fermentation, no bubbles. No active fermentation, no bubbles.
1.0	27625	14.8	No active fermentation, no bubbles. No active fermentation, no bubbles.

The following curve has been plotted to show graphically the number of cells in each tube :—



Curve showing relation between strength of solution of sodium nitrate and the number of yeast cells developed after 7 days. Ordinates express the number of yeast cells, abscissae the osmotic pressure of the solution in millimetres of mercury. Original number of yeast cells in each strength of solution was 18.

It is of interest to note that the most active production of new cells does not take place in the solution of least osmotic strength but in a solution of pressure equal to that of 6067 mm. of mercury. This strength agrees closely with that of the normal yeast juice as was shewn in a previous communication (6).

From that point onwards increase in osmotic strength of the solution results in a progressively decreasing rate of reproduction until in a solution of osmotic pressure equivalent to 19829 mm. of mercury the cells fail to reproduce at all. This is approximately the pressure at which the protoplasm ceases to exercise any pressure on the

cell-wall, as was shown in the communication to which reference has been made (6). In still greater concentrations of solution the yeast not only fails to reproduce but is actually killed, the number of cells being less than when the experiment was set up.

#### SUMMARY

1. The neutral salts, sodium and potassium chlorides and nitrates, fail to exercise any marked inhibiting action on the fermentative activity of yeast when added to sugar solution in strengths of 0.1, 0.01, 0.001 and 0.0001 gram-molecular.

2. Hydrochloric and nitric acids of 0.1 gram-molecular strength entirely prevent fermentation. In the weaker strengths they exercise depressing action.

3. Sodium and potassium hydroxides of 0.1 gram-molecular strength entirely prevent fermentation. The weaker solutions 0.01 and 0.001 depress the action, while 0.0001 affects the process but little.

4. Hydrochloric and nitric acids in strength 0.1 completely inhibit reproduction of the yeast and indeed are fatal to the cells in some degree. The lower concentrations permit reproduction of the cells.

5. In all strengths of the neutral salts employed the cells reproduce actively.

6. The hydroxides are to some extent lethal at a concentration of 0.1 gram-molecular and at this strength prevent any reproduction. In the lower concentrations reproduction takes place (except in 0.01 potassium hydroxide).

7. The effect on the average size of the cell in the case of 0.1 hydrochloric acid is very marked, shrinkage of the diameter to that of the dead cell taking place.

8. In a series of cultures containing sodium nitrate of all strengths differing by unity in the first place of decimals from 1.0 to 0.1 gram-molecular, the greatest rate of reproduction was found in 0.2. From this stage increasing strengths of the salt depressed



the reproductive activity down to 0.7, where no reproduction took place. In greater strengths the solutions proved lethal to some of the cells, so that a smaller number of cells was found at the end of the experiment than at the beginning.

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## ON THE PHOSPHORUS PERCENTAGE OF VARIOUS SAMPLES OF PROTAGON

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*(Received May 9th, 1907)*

In the most recent contribution to the protagon question Posner and Gies<sup>1</sup> have on the basis of a careful experimental investigation confirmed their conclusion that protagon is not a well-defined chemical substance, but a mixture of various substances, differing widely in the amount of phosphorus they contain and in their solubility in alcohol and ether. Although the results on which they base their view are supported by a wealth of analytical data, the interpretation which they put upon these results is open to criticism. A more detailed discussion of this subject must be reserved for a future occasion.

In this communication we intend to bring forward some positive evidence in favour of the view that protagon is an individual substance and not a mixture. It must be pointed out, however, that a final and decisive proof of the homogeneous nature of a substance, even of a less complex nature than protagon cannot be brought until the substance in question can be synthesized. In default of this the constancy of its physical and chemical properties is generally considered to be sufficiently trustworthy evidence. In order to definitely deprive a substance like protagon, possessing characteristic physical and chemical properties, of its existence as a chemical individual, it is necessary to isolate and identify the various substances constituting the mixture—a task which in view of the difference in the physical and chemical properties of these substances should be a comparatively easy one.

1. Posner and Gies, *Journal of Biological Chemistry*, Vol. I, p. 59, 1905.

Wörner and Thierfelder<sup>1</sup> have indeed claimed to have isolated a phosphorus-free substance from protagon which they called cerebron. In a former paper<sup>2</sup> the similarity of this substance with Gamgee's pseudocerebrin was pointed out.

The conclusion that these two substances were identical was confirmed by a letter from Professor Gamgee containing some valuable new data, which was received a few weeks after the publication of the paper referred to. With his permission we give here the paragraph referring to this subject. 'When Thierfelder's paper on cerebron appeared, I at once identified his body as my pseudocerebrin. The absolute identity of my body with his is proved not merely by the concordance of the ultimate organic analysis made by him and by me but by the result of the determination of the melting point. Unfortunately I did not publish these, but on referring to my laboratory note-book of this period, I find bearing the date July 11th, 1879, as a result of observations on the melting point of pseudocerebrin (in capillary tubes),

Becomes slightly orange at 197° C.

Melts ... .. at 210° C.

'Wörner and Thierfelder obtained identical results. They say, "Erst bei 200° wird es leicht gelblich. Bei langsamen Erhitzen bei 209°, bei schnellem bei 212° schmilzt es zu einer klaren gelblichen Flüssigkeit." No closer concordance between melting point determinations could be obtained and it is obvious not only that we have worked on the same body, but that, although I made no pretensions in reference to the purity of pseudocerebrin, the body I analysed was identical with and as pure as Thierfelder's.'

In a recent paper Thierfelder<sup>3</sup> has frankly acknowledged the identity of cerebron and pseudocerebrin.

According to Gamgee<sup>4</sup> pseudocerebrin exists together with protagon and is extracted together with protagon from the brain.

1. Wörner und Thierfelder, *Zeitschrift für physiolog. Chemie*, Vol. XXX, p. 542, 1900.

2. Cramer, *Journal of Physiology*, Vol. XXXI, p. 31, 1904.

3. Thierfelder, *Zeitschrift für physiolog. Chemie*, Vol. XLIII, p. 21, 1904.

4. Gamgee. *Text-book of Physiological Chemistry*, Vol. I, London, p. 44, 1880.

Being less soluble in alcohol than protagon, Gamgee was able to separate it from protagon by simple recrystallisation. If his view is correct, it is possible to remove pseudocerebrin entirely from the phosphorised matter, which can be obtained as a definite chemical compound of a constant phosphorus percentage. According to Posner and Gies pseudocerebrin is one of the substances of which the mixture called protagon is made up. Every sample of protagon that has ever been prepared, even Gamgee's protagon, contains, according to them, considerable quantities of pseudocerebrin. If their view is correct, the phosphorus percentage of samples obtained in repeated crystallisations or by different methods of preparation should not remain constant. Gamgee,<sup>1</sup> however, found that after repeated recrystallisation the phosphorus content of the protagon remained constant. Posner and Gies have recrystallised protagon as often as ten times. They found the phosphorus percentage of the crystals separating out (0.93 per cent.) and of the substance in the mother liquor (1.02 per cent.) to be almost identical<sup>2</sup>—surely the most conclusive proof of the homogeneous nature of the substance they were dealing with. The significance of this result does not seem to have been recognised by the authors. According to them even this sample contained considerable quantities of pseudocerebrin as evidenced by the effect of their method of fractionation.

There is further the evidence<sup>3</sup> that by using a method quite different from that of previous observers and entailing the use of relatively high temperatures, a substance was extracted from brain which was identical with the protagon of previous workers. If we accept the view of Gies and his collaborators and consider protagon to be a mixture of substances varying in their solubility and in their phosphorus percentage, we should expect to obtain different mixtures by extracting brain tissue by different methods, especially by using different solvents.

In order to apply solvents such as chloroform to brain tissue it

1. Gamgee, *Text-book of Physiological Chemistry*, p. 428.

2. Posner and Gies, *loc. cit.*, p. 107.

3. Cramer, *loc. cit.*



is necessary to remove the water. This we have done by following the method employed by Bünz<sup>1</sup> for the preparation of cholesterin. Fresh ox brains, after having been passed through a mincing machine, were rubbed up in a mortar with anhydrous sodium sulphate, until a dry powder was formed. This powder was extracted with cold ether until all the lecithin and cholesterin had been removed. The residue was used for the preparation of protagon by adding the various solvents and extracting with the boiling solvent for one to two minutes. The phosphorus percentage of the various samples was estimated by fusing with soda saltpetre mixture and weighing the phosphoric acid as magnesium pyrophosphate.

*Protagon A.*—The dry powder was extracted with 1500 c.c. of boiling absolute alcohol. The crystals separating out were recrystallised three times, the second and third recrystallisation were submitted to analysis.

Second recrystallisation 1.34 per cent. P.

Third recrystallisation 1.07 per cent. P.

*Protagon B.*—A second sample of protagon was prepared in exactly the same way. The second recrystallisation gave on analysis—

Second recrystallisation 1.25 per cent. P.

One portion of this sample was recrystallised out of boiling absolute alcohol.

Third recrystallisation 1.05 per cent. P.

*Protagon C.*—Another portion was recrystallised out of boiling glacial acetic acid and contained 0.96 per cent. P.

*Protagon D.*—The dry powder was extracted with 1500 c.c. of boiling methylalcohol. The substance separating out on cooling was recrystallised three times out of boiling methylalcohol. The analysis of these samples gave the following figures :—

Second recrystallisation 0.94 per cent. P.

Third recrystallisation 0.97 per cent. P.

*Protagon E.*—A sample of protagon was prepared in the manner described for samples A and B. After one recrystallisation it contained 1.22 per cent. P. It was recrystallised once more out of methylalcohol

1. Bünz, *Zeitschrift für physiolog. Chemie*, Vol. XLVI, p. 47, 1905.

in the following manner : a small quantity of the solvent, which was not sufficient to dissolve all the protagon, was added first and decanted after having kept the solution boiling for one minute. The same process was repeated with another small quantity of methylalcohol. The crystals separating out in these two fractions gave on analysis—

First Fraction 1·14 per cent. P.  
Second Fraction 1·05 per cent. P.

*Protagon F.*—The dry powder was extracted with 500 c.c. of boiling chloroform. After cooling 1500 c.c. of ether were added. A white precipitate formed, which was analysed without further purification, as only small quantities could be obtained in this way. It contained 1·18 per cent. P.

On cooling the chloroform extract nothing separated out. After evaporating the bulk of the chloroform in vacuo, a viscous semi-fluid mass remained, which did not crystallise on standing.

None of the methods can be recommended for practical use in the preparation of protagon. Of all the solvents used absolute alcohol gave the best results. Nor does the method of removing the water by mixing the brain pulp with anhydrous sodium sulphate offer any advantage as it increases too much the bulk of the matter to be extracted. A better way of removing the water is to treat the brain pulp with cold alcohol and ether. In this way a dry powder is obtained and at the same time superfluous matter (lecithin, cholesterolin, etc.) is removed, so that the bulk of the matter is greatly reduced. Details of this method will be given in a later paper.

The results of our analyses can be seen more clearly from the following table.

Solvent ... ..	Aethyl-Alcohol		Glacial Acetic	Methyl-Alcohol		Chloroform
Sample of Protagon ... ..	A	B	C	D	E	F
Crude crystalline product ... ..	—	—	—	—	—	1·18
First recrystallisation ... ..	—	—	—	—	—	—
Second recrystallisation ... ..	1·34	1·25	—	0·94	{ 1·14 1·05	—
Third recrystallisation ... ..	1·07	1·05	0·96	0·97	—	—

It will be seen that the phosphorus percentage of these various samples of protagon, extracted by different solvents, is remarkably constant. Comparing only the most highly purified products, A, B, C and D, the greatest difference is = 0.11 between sample A and sample C. Sample F, prepared by precipitation with ether, is of special interest as, according to Gies,<sup>1</sup> protagon contains a considerable quantity of an ether soluble substance, poor in phosphorus. If this is correct, the method employed for Sample F should yield a substance richer in phosphorus than the other samples of protagon. This was not found to be the case.

A sample of protagon which was prepared by the coagulation method (heating the brain emulsion with a concentrated sodium sulphate solution in the boiling water bath and extracting the coagulum with boiling absolute alcohol) contained after three recrystallisations 1.07 per cent. P.

The figures which have been obtained by previous workers may be given here for comparison.

Liebreich <sup>1</sup>	...	...	...	...	1.23 per cent. P.
Gamgee <sup>2</sup>	...	...	...	...	1.07 per cent. P.
Baumstark <sup>3</sup>	...	...	...	...	1.02 per cent. P.
Kossel and Freitag <sup>4</sup>	...	...	...	...	0.97 per cent. P.
Ruppel <sup>5</sup>	...	...	...	...	1.13 per cent. P.
Cramer <sup>6</sup>	...	...	...	...	1.07 per cent. P.
Posner and Gies <sup>7</sup> (Gamgee's method)					0.93 per cent. P.
(Cramer's method)					0.93 per. cent P.

1. Liebreich. *Liebig's Annalen*, Vol. CXXXIV, p. 29, 1864.

2. Gamgee. *Text-book of Physiological Chemistry*, p. 428.

3. Baumstark. *Zeitschrift für physiolog. Chemie*, Vol. IX, p. 145, 1888.

4. Kossel and Freitag. *Ibid.*, Vol. XVII, p. 431, 1892.

5. Ruppel. *Zeitschrift für Biologie*, Vol. XXXI, p. 86, 1892.

6. Cramer. *Loc. cit.*

7. Posner and Gies. *Loc. cit.*, pp. 97, 109.

1. Posner and Gies, *loc. cit.*, p. 111.

## CONCLUSIONS

The close agreement between the phosphorus percentage of various samples of protagon prepared by the most diverse methods is strong evidence in favour of the view that protagon is an individual substance of a well-defined chemical composition. Even more conclusive evidence is afforded by the observation of Posner and Gies, that after ten times repeated crystallisation the protagon crystals separating out have the same phosphorus percentage as the substance in the mother liquor.

The view that protagon is a mixture of substances differing in their solubility and in their phosphorus contents is not compatible with these results and cannot be accepted until the substances constituting the mixture have been isolated.

The expenses of this research, which was carried out during the year 1906, were defrayed by a grant from the Moray Fund.



## OBSERVATIONS ON THE BEHAVIOUR OF OPSONIN AND SERUM PROTEIDS DURING PRESSURE FILTRATION

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Martin (1) in 1896 introduced a method for separating colloids from crystalloids in solutions containing both. It consisted in filtering the fluid through a Pasteur-Chamberland filter, the pores of which had been blocked up with gelatine, under a pressure of forty to one hundred atmospheres. He found that if fresh serum or white of egg were passed through such a filter the filtrate was clear, colourless, and absolutely free from proteid. The crystalloids in solution on the other hand passed through the filter at the same rate as water.

Since the publication of the above paper, the gelatine filter has been employed on various occasions for the purpose of observing the behaviour of substances of physiological and pathological interest, when a solution containing such was passed through the filter.

Martin and Cherry (2) found that snake venom and diphtheria toxin passed through the filter and appeared in the filtrate, whereas the antibodies were retained.

Craw (3) made use of the gelatine filter in his work on the physical chemistry of the toxin-antitoxin reaction; he observed that while megatherium lysin passed through the filter, the megatherium antilysin was retained.

In the following experiments an attempt was made to observe the behaviour of opsonin when serum was forced through a filter similar in nature to the gelatine filter.

The method employed was that described by Martin *with the important exception that no gelatine was used to impregnate the filter*. A Pasteur-Chamberland filter was securely fixed into an iron jacket the inner surface of which was tinned. The upper end of this outer case, which served to hold the serum to be filtered, was closed and connected with a gas cylinder, which itself communicated with an engine by which it could be charged to a pressure of 200 to 300 atmospheres. The Pasteur-Chamberland candle used in the following observations was of a rather fine texture, its pores being comparatively small.

Fresh pig's serum was introduced into the metal case, and by means of a pressure of forty to fifty atmospheres was forced through the porcelain candle and the filtrate collected as it dropped through below.

At first little difference was to be noticed between the normal serum and the filtrate, which passed through the filter fairly quickly. After a short time, however, a very distinct difference was to be noticed, the filtrate, which escaped much more slowly, was observed to be clearer and more watery than normal serum. Ultimately the flow at the outlet of the filter became so slow that a pressure of eighty atmospheres sufficed to cause an escape of only one or two drops per minute, and sometimes the filtration was so retarded that only one drop escaped every three or four minutes.

Systematic examination of the filtrate showed that it contained less and less proteid as the filtration proceeded, until in the later stages it was found to be quite free from proteid.

Thus it is evident that by forcing serum through this fine porcelain candle one had been able to fill up the pores by the large proteid molecules of the serum, so that in a short time the filter had been converted into what was practically a gelatine filter.

Samples of the filtrate were taken at different periods as it escaped from the filter and their opsonic action compared with the normal serum and also with the contents of the outer case of the filter after about one-third of its volume had been forced through the filter.

The following are some of the experimental results :—

*Experiment I.*—In this experiment pig's serum, staphylococcus albus and human leucocytes were used.

	Number of bacteria phagocytosed by 100 leucocytes					
Control Serum ... ..	...	...	...	...	...	446
Filtrate after 5 minutes ... ..	...	...	...	...	...	430
Filtrate after $\frac{1}{4}$ hour ... ..	...	...	...	...	...	58
Filtrate after 1 hour ... ..	...	...	...	...	...	80
Specimen of the serum taken from inside the metal jacket when about one-third of its volume had passed through the filter ... ..	...	...	...	...	...	458

*Experiment II.*—Here also fresh pig's serum, staphylococcus albus and human leucocytes were used.

	Number of bacteria phagocytosed by 100 leucocytes					
Control ... ..	...	...	...	...	...	920
Filtrate after 5 minutes ... ..	...	...	...	...	...	938
Filtrate after 2 hours ... ..	...	...	...	...	...	164
Sample of serum taken from inside the metal jacket when about one-third of its volume had passed through the filter ... ..	...	...	...	...	...	916

*Experiment III.*—In this case B. Coli were used instead of staphylococcus albus.

	Number of bacteria phagocytosed by 100 leucocytes					
Control ... ..	...	...	...	...	...	414
Filtrate after 1 hour ... ..	...	...	...	...	...	60
Filtrate after $2\frac{1}{2}$ hours ... ..	...	...	...	...	...	32
Sample of serum taken from inside the metal jacket when about one-third of its volume had passed through the filter ... ..	...	...	...	...	...	460

*Experiment IV.*—Here Tubercle bacilli were substituted for staphylococcus albus.

	Number of bacteria phagocytosed by 100 leucocytes					
Control ... ..	...	...	...	...	...	396
Filtrate after 5 minutes ... ..	...	...	...	...	...	429
Filtrate after 1 hour ... ..	...	...	...	...	...	34
Filtrate after 2 hours ... ..	...	...	...	...	...	42
Sample of serum taken from inside the metal jacket when about one-third of its volume had passed through the filter ... ..	...	...	...	...	...	440

From these results it is apparent that, if any, only the merest trace of opsonin passed through the filter after it had been working sufficiently long for its pores to become filled up by the proteids of the serum. The enormous decrease in the opsonic power of the filtrate as compared with normal serum is even more pronounced, when the fact is recalled, that considerable dilution of normal serum by either '9 per cent. saline solution or by heated serum suffices to reduce its opsonic action, when measured by Wright and Douglas' method, to a slight extent only. Wright and Douglas (4) found that normal serum can be diluted twenty-four fold with only a comparatively slight lessening of its opsonic power when compared with undiluted normal serum. Then again the slight amount of spontaneous phagocytosis, which occurs when leucocytes washed free from serum are incubated with bacteria in the entire absence of serum, has to be taken into account when one is considering the significance of the above figures.

When, after about one-third of the serum had passed through the filter, the contents of the jacket were examined it was found that besides the comparatively unchanged serum, the opsonic power of which was given in the above experiments, there was a gelatinous substance which was adherent to the porcelain candle. This consisted of the contents of the serum which could not pass through the filter. An attempt was made to estimate the opsonic activity of this substance as compared with the normal serum. Beyond, however, ascertaining that it contained a considerable amount of opsonin one was unable to proceed, as, owing to its gelatinous nature, it was quite impossible by the ordinary method of Wright and Douglas to prepare specimens that were at all comparable with those made from normal serum.

In order to clean the filter, the candle was removed and the adherent jelly washed off, and then after being allowed to dry thoroughly in an oven the porcelain was heated red hot by means of a blow-pipe. By this means all organic substances in the pores of the candle were removed.

Since opsonins did not pass through a filter of this nature it seemed probable that they were of the nature of proteid.



The following experiments were performed to ascertain whether opsonin in normal serum was dialysable. Ten cubic centimetres of fresh serum were placed in a parchment tube and this was immersed in a somewhat wider glass tube containing an equal quantity of normal saline solution. At the same time a quantity of serum was put up in a Wright's capsule to serve as a control.

After allowing dialysis to proceed for twenty-four hours and forty-eight hours respectively, samples were drawn off from the inner tube containing the serum and from the outer tube containing the '9 per cent. NaCl solution into which the serum was being dialysed. The opsonic power of these specimens was then compared with the control serum. The following are the results:—

*Experiment I.*—Pig's serum, *Staphylococcus albus* and human leucocytes were used.

						Number of bacteria phagocytosed by 100 leucocytes
A. After allowing dialysis to proceed for 24 hours—						
Normal pig's serum	...	...	...	...	...	483
Dialysed pig's serum	...	...	...	...	...	470
'9 per cent. saline solution into which the serum was dialysed	...	...	...	...	...	4
B. After allowing dialysis to proceed for 48 hours—						
Normal pig's serum	...	...	...	...	...	268
Dialysed pig's serum	...	...	...	...	...	298
'9 per cent. saline solution into which the serum was dialysed	...	...	...	...	...	2

*Experiment II.*—In this case *Tubercle bacilli* were substituted for *Staphylococci*.

						Number of bacteria phagocytosed by 100 leucocytes
A. After allowing dialysis to proceed for 24 hours—						
Normal pig's serum	...	...	...	...	...	229
Dialysed pig's serum	...	...	...	...	...	241
'9 per cent. saline solution into which the serum was dialysed	...	...	...	...	...	8
B. After allowing dialysis to proceed for 48 hours—						
Normal pig's serum	...	...	...	...	...	326
Dialysed pig's serum	...	...	...	...	...	297
'9 per cent. saline solution into which the serum was dialysed	...	...	...	...	...	9

We have from these figures conclusive proof that opsonins are not dialysable.

Simon Tamar and Bispham (5) also find that opsonins are not dialysable, and that they are carried down with euglobulin when serum is treated by half saturation with ammonium sulphate. These observations also serve to support the view that opsonin found in normal serum is of the nature of a proteid.

In conclusion I desire to express my indebtedness to Professor Moore for his advice, and assistance in connection with the use of the filter.

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## HEAT RIGOR IN VERTEBRATE MUSCLE

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The experiments described in the following paper were commenced in the winter of 1905-06 by Dr. Kenny. His work was not brought to a conclusion, though the results he arrived at were to a great extent in conformity with those about to be described.

I have to express my thanks to Professor Vincent for the use of his laboratory and for his assistance throughout the course of the work.

The generally received view as to the chemistry of muscle (so far at any rate as concerns a study of the proteins contained in saline extracts of the tissue), is that we have to deal with two muscle proteins, viz., paramyosinogen (V. Fürth's myosin) and myosinogen (V. Fürth's myogen).<sup>1</sup> These are probably identical or closely related in both striped and unstriped muscle.

The earliest attempt to which a detailed reference need be made to study the protein constitution of muscle by means of a graphic record was that of Brodie and Richardson.<sup>2</sup> These observers using

1. References upon the more purely chemical aspect of the question are as follows:—Halliburton, *Text-Book of Physiology*, ed. by Schäfer, p. 99, 1898; Kühne, *Lehrbuch d. Physiol. Chem.*, S. 272; Untersuch. u. das Protoplasma, Leipzig, 1864; Halliburton, *Journ. of Physiol.*, Vol. VIII, p. 133, 1887; V. Fürth, *Arch. f. exper. Path. u. Pharmacol.*, Leipzig, Vol. XXXVI, S. 231, 1895, Vol. XXXVII, S. 389, 1896; Stewart and Sollman, *Journ. of Physiol.*, Vol. XXIV, p. 427, 1899; Velich, *Centralbl. f. Physiol.*, Vol. XII, p. 351, 1898; Vincent and Lewis, *Journ. of Physiol.*, Vol. XXVI, p. 445, 1901; Vincent, *Hoppe-Seyler's Zeitschr. f. physiol. Chem.*, Vol. XXXIV, S. 417, 1902.

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the living sartorius of the frog found four distinct contractions. 'The first contraction commences at  $28^{\circ}$  C., its rate is much accelerated at  $32^{\circ}$  C., and it is completed shortly after  $40^{\circ}$  C. is reached. It is then followed by a fairly rapid and extensive relaxation.

The second contraction commences at  $44^{\circ}$  C., its main effect is produced at a slightly higher temperature and it is completed at about  $40^{\circ}$  C.

The third begins at  $55^{\circ}$  C. and is complete soon after  $60^{\circ}$  C.

The fourth commences at  $63^{\circ}$  C.'

They give a tracing in support of these conclusions which, however, to me, seems not very convincing. It appears, however, to show three contractions; the fourth, they admit, is not very prominently marked. Some criticisms of the apparatus employed by these observers have been offered by Vernon<sup>1</sup> and certainly the results obtained (so far as we can interpret the tracing) do not correspond to those obtained by the present writer. In mammalian muscle these observers found only two contractions, but instead of considering these, as seems to be actually the case, to be due, respectively, to muscle protein and to connective tissue elements, they attribute them to the two muscle proteins.

Vernon<sup>2</sup> performed an elaborate series of experiments upon the heat rigor of cold-blooded animals. In the striped muscle he found three contractions, but the highest of these was certainly due to blood. In unstriped muscle the first contraction, he states, is absent.

Vincent and Lewis<sup>3</sup> found also two contractions both in mammalian and amphibian muscle, but they refer also to 'a tendency to contract at about  $56^{\circ}$  C.' in the case of mammalian tissue. The first of these two contractions, they conclude, is due to the coagulation by heat of the protein substance present in the muscle fibre during life, the second to changes in the connective tissue elements in the muscle. Curiously enough, they, like Vernon, could not record the first contraction in amphibian unstriped muscle. There can be no

1. *Journ. of Physiol.*, Vol. XXIV, p. 241.

2. Vernon, *loc. cit.*

3. Vincent and Lewis, *loc. cit.*



doubt from my own experiments that this was an error of observation, and was due to one or other of two causes—either the tissue was dead (rigored), and so the first contraction would be absent (*vide infra*), or the material used was so delicate that the counterpoise employed was too great to allow of a contraction, and caused relaxation instead. The figure they give, Fig. 9 of their paper, seems to confirm this latter hypothesis. These observers prove conclusively that the second contraction is due to connective tissue.

Brodie and Richardson state that ‘a muscle which has passed into rigor mortis, when treated as in our experiments, will give heat contractions, though, as we shall show in a paper we hope to publish shortly, the form of these contractions is greatly affected by the stage which the muscle has reached in rigor mortis.’ I have not been able to find any further publication on this subject by the authors in question, but Vincent and Lewis state definitely that a muscle which has gone into complete rigor mortis gives no trace of contraction on being heated to about  $47^{\circ}\text{C}$ . ‘The only change observed in this case is the contraction at about  $63^{\circ}\text{C}$ . (due as we show to changes in the connective tissue).’<sup>1</sup> They give a tracing in support of this statement, and, as will be seen below, it is entirely borne out by my own experiments.

Since the paper of Vincent and Lewis a communication by Inagaki has appeared on this same subject, but which I have been unable to consult.<sup>2</sup>

In a communication on heat contraction in nerve Brodie and Halliburton<sup>3</sup> have incidentally made some observations on the corresponding phenomena in muscles. These observers appear to be satisfied that the successive steps of heat rigor both in nerve and muscle occur at temperatures corresponding to the points of coagulation of the proteins that can be extracted by saline solutions from the tissues in question.

1. Vincent and Lewis, *loc. cit.*

2. *Zeitschr. f. Klin. Med.*, Vol. XLVIII, pp. 313-339, 1906. From a reference, however, to this work by V. Frey (*Physik.-Med. Gesellsch. z. Würzburg. Sitz. Vom VIII, Nov., 1906; Munch. Med. Woch.*, Jan. 29, 1907) it seems that this author has reached conclusions very similar to my own.

3. *Journ. of Physiol.*, Vol. XXXI, p. 473, 1904.

## RESULTS OF HEAT RIGOR EXPERIMENTS

Little need be said about the apparatus employed. The method adopted has been practically identical with that previously used by Brodie and Richardson,<sup>1</sup> Vernon,<sup>2</sup> and Vincent and Lewis.<sup>3</sup>

‘An iron bar clamped in a stand at one end, and bent down at the other into a beaker, has a small metal hook soldered to it. This forms the attachment at the lower end of the piece of muscle under investigation. The upper end is suspended by another hook attached by a very fine copper wire to the recording lever. The resistance employed was in some cases a fine spiral spring, in others a weight passing over a small frictionless pulley. The waxed thread employed by Brodie and Richardson was shown to be disadvantageous by Vernon, who, however, left a small length of this in the apparatus he employed. We have completely eliminated any defect from this source by using metal throughout. The metal hooks have, moreover, a great advantage in point of simplicity of manipulation.

‘Our recording lever has, as a rule, magnified the extent of change of length in the muscle ten times.

‘We have always taken care that the load should be small, so as to obviate any fallacy from increased extensibility on raising the temperature. Further, to completely eliminate other sources of instrumental error, and to check the results obtained by the above apparatus, we have performed a series of experiments in which we have taken long strips of the muscle and directly observed the changes in length at the different temperatures. The muscle was suspended in a large beaker of normal saline solution with a light load attached, and placed by the side of it was a millimetre scale. The changes in length at different temperatures could then be readily and directly observed.’<sup>4</sup>

In the present series of experiments all these precautions have been again taken. In order to guard against any possible fallacy

1. *Journ. of Physiol.*, Vol. XXI, p. 353, 1897, and *Phil. Trans.*, Vol. CXCI, p. 127, 1899.

2. *Journ. of Physiol.*, Vol. XXIV, p. 239, 1899.

3. *Loc. cit.*

4. Vincent and Lewis, *loc. cit.*

arising from inertia or undue weight of moving parts, rather than reduce these to their smallest dimensions, I have preferred to bring about the desired result by using only large and powerful strips of muscles. In some experiments I have employed a device for recording the precise vertical movement of the muscle, without the use of the magnifying lever. (See Fig. 2.)

In many experiments the metal attachments at the ends of the strip of muscle have been insulated, and attached to wires leading from the secondary coil of an inductorium, so that during the course of a heat contraction the irritability of the muscle could be readily tested. (See Figs. 1 and 7).

In experimenting thus with mammalian muscle I have taken the material direct from the living animal so as to be reasonably certain that the loss of irritability was due to the change of temperature, and was not spontaneous owing to lapse of time after removal from the body.

1. *Effects of temperature on mammalian striped and unstriped muscle, living.*—On testing mammalian striped or unstriped muscle I find that the contractions obtained differ according as to whether the tissue is living at the beginning of the experiment or is dead. On gradually heating from room temperature up to  $70^{\circ}$  or  $80^{\circ}$  C. we find in living muscle two contractions. *First* at about  $47^{\circ}$  C. ( $46^{\circ}$  to  $50^{\circ}$ ). *Second* at about  $62^{\circ}$  C. ( $61^{\circ}$  to  $64^{\circ}$ ). (See Figs. 1 and 2).

As mentioned above Vincent and Lewis describe three contractions, viz., (1)  $45^{\circ}$  to  $49^{\circ}$ , (2)  $55^{\circ}$  to  $58^{\circ}$ , (3)  $61^{\circ}$  to  $64^{\circ}$  C. In regard to the second contraction they note:—‘A very faint indication of contraction or, more frequently, rather a cessation of relaxation at about  $55^{\circ}$  to  $58^{\circ}$  C.’ Professor Vincent tells me he is now doubtful about this result, and the tracings given in his paper with Mr. Lewis do not lend much support to the view that a contraction occurs at this temperature.<sup>1</sup>

*Causes of the first contraction.*—In the opinion of the present writer this contraction is due to the coagulation by heat of the complex protein present in muscle fibre at the moment of death. Stewart and Sollmann<sup>1</sup> suggest ‘that paramyosinogen does not exist as such

1. *Journ. of Physiol.*, Vol. XXIV, p. 427, 1899.

in living muscle,' but, as pointed out by Vincent and Lewis, if either of the described muscle proteins (which can be separated by the heat coagulation method from a saline extract<sup>1</sup>), be absent at the moment of death—it is the one coagulating at 56° C. and not that which comes down at 47° C. These authors further point out that, 'The fact that the contraction occurs regularly at this temperature in the heat-rigor method (in which the muscle is in the best possible state for investigation, and in which its constituents are probably less injured or changed than in any extraction method) seems strong evidence in favour of this view.'

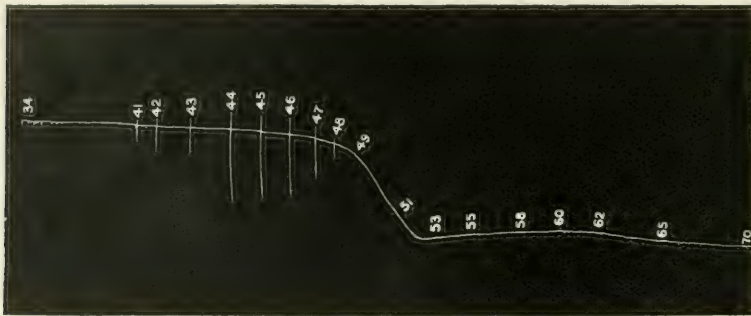


FIG. 1.—Dog's striped muscle. Note contractions due to electric stimuli, ceasing at 48° C.

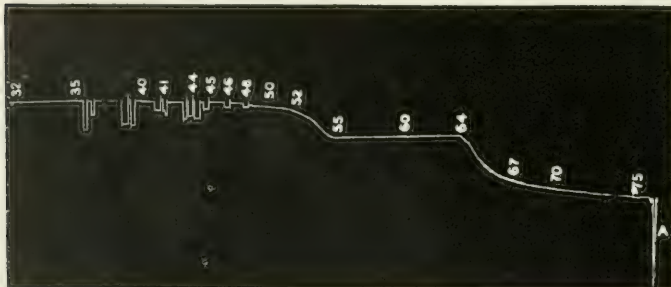


FIG. 2.—Dog's striped muscle, living. Apparatus used in this experiment shows length of contraction without magnifying. A large strip of muscle was used. A represents length of muscle at end of experiment.

1. Halliburton, *Journ. of Physiol.*, Vol. VIII, p. 133, 1887.



We may then provisionally conclude that the cause of the contraction at about  $47^{\circ}$  C. is due to the coagulation by heat of the muscle protein.

In the experiments in which I started with living muscle it has been found that loss of irritability occurs at or immediately after the temperature at which this first contraction begins.

*Cause of the Contraction at  $63^{\circ}$  C.*—This is due, as first clearly pointed out by Vincent and Lewis, to some change in the connective tissue elements of the muscle substance.<sup>1</sup> Tendon and skin show a marked contraction at this temperature.

#### EFFECTS OF HEAT RIGOR ON AVIAN MUSCLE

The results I have obtained point to the conclusion that the same contractions occur in avian as in mammalian muscle, and that the same modification is determined by the presence of rigor mortis or death of the muscle. The results are shown in Figs. 3 and 4.

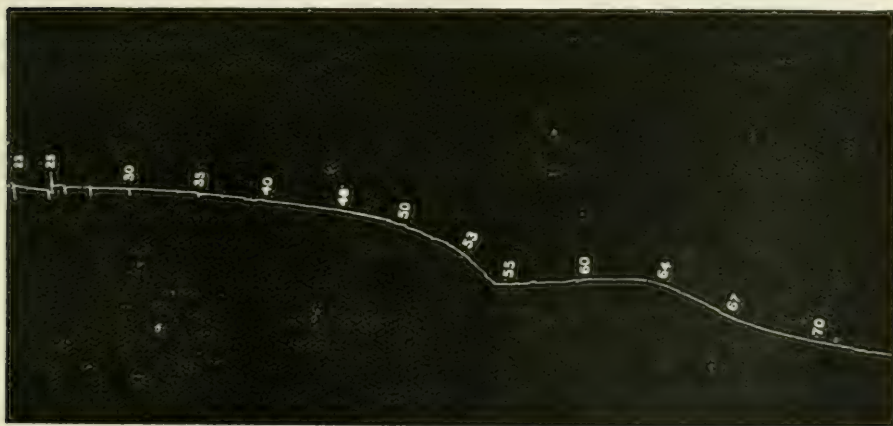


FIG. 3.—Pigeon's striped muscle, living.

1. Vincent and Lewis, *loc. cit.*

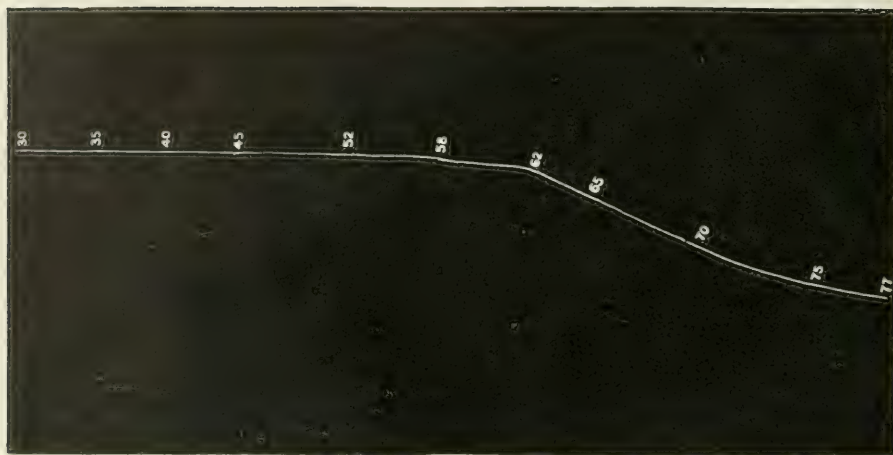


FIG. 4.—Pigeon's striped muscle, dead twenty-four hours. Rigor mortis present.

#### EFFECTS OF TEMPERATURE ON AMPHIBIAN STRIPED AND UNSTRIPED MUSCLE (LIVING)

On testing the striped muscle of frogs living at the beginning of the experiment, we find (1) a marked contraction at about  $39^{\circ}\text{C}$ . ( $38^{\circ}$  to  $41^{\circ}$ ); (2) a contraction at about  $50^{\circ}\text{C}$ . ( $49^{\circ}$  to  $51^{\circ}$ ). These results are shown in Fig. 5.



FIG. 5.—Frog's gastrocnemius, living. Note contractions due to electric stimuli up to  $42^{\circ}\text{C}$ .

In unstriated amphibian muscle the same two contractions occur, but in this case there is always considerable relaxation between the two contractions. (See Fig. 6.)

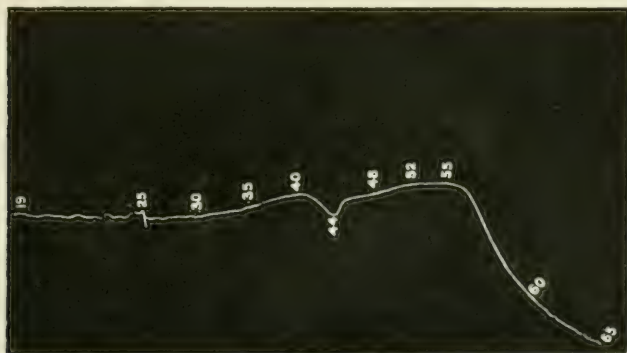


FIG. 6.—Frog's stomach, living. Marked relaxation after the first contraction is well shown in this tracing.

Thus it will be seen that in amphibian as well as mammalian muscle of both kinds living at the beginning of the experiment we get two contractions, and we may assume that in both cases the first is due to the muscle protein, the second to the connective tissue.

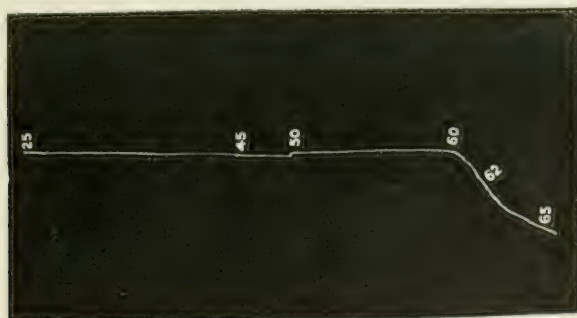


FIG. 7.—Rabbit's striped muscle, dead one day. Rigor mortis present.

It is stated by Vernon, as quoted above, that the  $37^{\circ}$  to  $40^{\circ}$  C. contraction is absent in amphibian unstriated muscle, and this was

the opinion also of Vincent and Lewis.<sup>1</sup> There can, however, be little doubt that this contraction occurs, as I have obtained it in five successive experiments, and it is well shown in Fig. 6. The only differences, then, between amphibian and mammalian muscle are (1) that the two contractions occur at a lower temperature in the former case than in the latter ( $47^{\circ}$  and  $62^{\circ}$  C. in mammalian and  $39^{\circ}$  and  $50^{\circ}$  C. in amphibian); (2) the marked relaxation after the first contraction in the case of amphibian muscle.

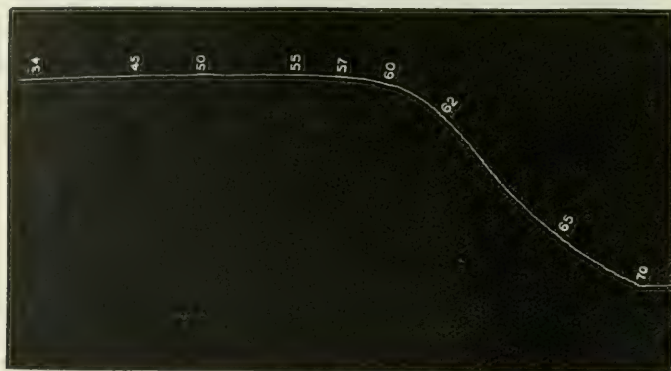


FIG. 8.—Rabbit's bladder, dead two days. Rigor mortis present.

#### HEAT CONTRACTIONS IN RIGORED MUSCLE

It has been stated<sup>1</sup> that 'if a muscle which has been passed into a state of rigor mortis be subjected to a rising temperature the above contractions are modified according to the stage the rigor has reached. When this is complete the lever writes a straight line until a temperature of  $63^{\circ}$  C. is reached, when it suddenly drops and falls off the drum.' My own experiments fully confirm this observation.

On gradually heating mammalian muscle, striped or unstriped, which is either in a state of rigor or has passed beyond this stage,

1. Vincent and Lewis, *loc. cit.*



we find that the first contraction, viz., at  $47^{\circ}\text{C}$ . never occurs. One contraction, and one contraction only, is obtained, viz., at about  $63^{\circ}\text{C}$ .

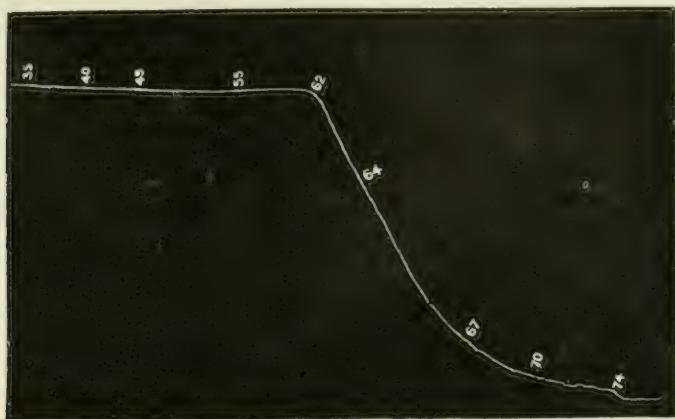


FIG. 9.—Dog's striped muscle. Rigor mortis present but not very pronounced.

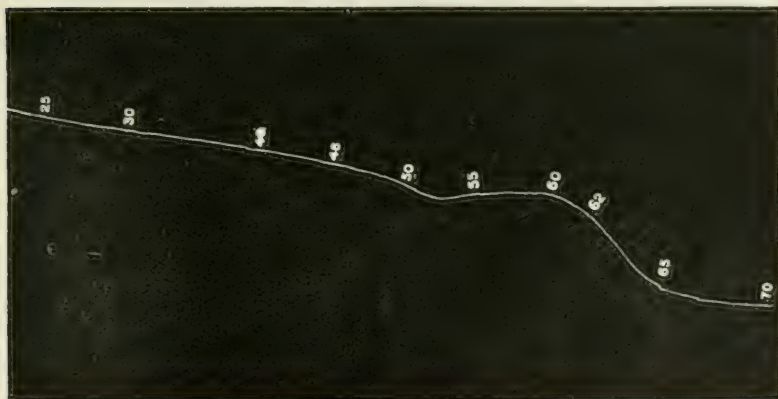


FIG. 10.—Rabbit's striped muscle, dead three hours. Rigor not yet present. No reaction to electric stimulus.

On similarly testing rigored or dead amphibian muscle, striped or unstriped, we find analogous conditions. The first contraction

(at  $38^{\circ}$  C.) does not occur. Here again we get one contraction only, viz., at about  $53^{\circ}$  C.

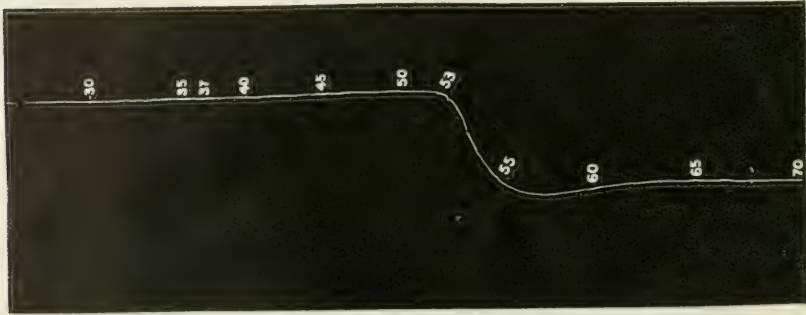


FIG. 11.—Frog's gastrocnemius, dead ten days.

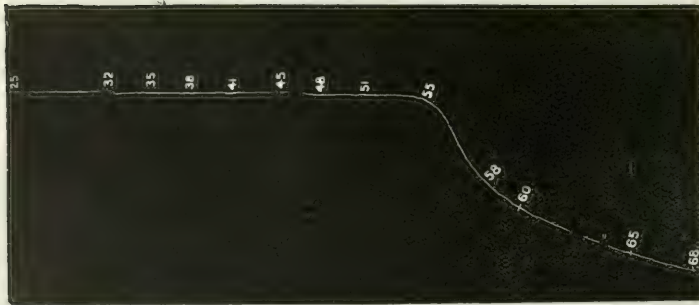


FIG. 12.—Frog's stomach, dead three days.

In the three groups of vertebrates, then, we obtain in rigor muscle only one heat contraction, due to effects upon connective tissue elements. That previous observers have not noted this in the case of frog's muscle is probably due to their having underestimated the lapse of time necessary to bring on rigor mortis in this animal. In many instances we have obtained the first contraction when the animal has been lying in saline solution apparently dead for several days, but in all these cases, on testing the matter, we have found that the muscles would respond to an electrical stimulus. If still further time

be allowed to elapse, so that no reaction will occur on stimulation, then the muscles will give only one heat contraction, viz., that due to connective tissue at  $52^{\circ}$  C. Further, if the animals are killed by chloroform, rapid rigor, as is well known, will ensue, and in the muscles from an animal so killed we get only the  $53^{\circ}$  C. contraction.

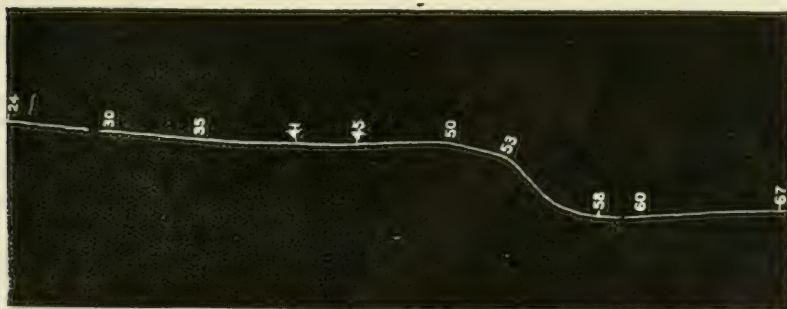


FIG. 13.—Frog's gastrocnemius, animal killed by chloroform. Rigor mortis very marked.

We have seen that when muscles are in a state of complete rigor mortis at the beginning of the experiment the first (protein) contraction is entirely absent. It must now be added that when the muscle is in a state of partial rigor a modified or slight degree of contraction may be expected at about  $47^{\circ}$  C.—see Fig. 10 and compare with Figs. 9 and 7 and 8.

The usual teaching in regard to this subject may be illustrated by the following quotation from a recent text-book.<sup>1</sup> 'It is found that the first shortening occurs at the coagulation temperature of paramyosinogen ( $47^{\circ}$  to  $50^{\circ}$  C.), and if the heating is continued a second shortening occurs at  $56^{\circ}$  C., the coagulation temperature of myosinogen. If frog's muscles are used there are three shortenings, viz., at  $40^{\circ}$ ,  $47^{\circ}$  and  $56^{\circ}$  C.'

It will readily be seen how widely this differs from the results detailed above. It is particularly to be noticed that no mention is made of any connective tissue shortening.

1. Halliburton, *Essentials of Chemical Physiology*, p. 177, London, 1904.

## SUMMARY OF CONCLUSIONS

1. Both striped and unstriped mammalian living muscle on being subjected to a gradually rising temperature undergo two marked and comparatively sudden contractions: First, at about  $47^{\circ}\text{C}$ . ( $46^{\circ}$  to  $50^{\circ}$ ); Second, at about  $62^{\circ}\text{C}$ . ( $61^{\circ}$  to  $64^{\circ}$ ).

2. Striped and unstriped amphibian muscle under similar conditions likewise undergoes two contractions: First, at about  $39^{\circ}\text{C}$ . ( $38^{\circ}$  to  $40^{\circ}$ ); Second, at about  $50^{\circ}\text{C}$ . ( $49^{\circ}$  to  $51^{\circ}$ ).

3. Any muscle, striped or unstriped, mammalian, avian, or amphibian, taken from an animal in a state of rigor mortis, or from one which has passed beyond this stage, does not show on being subjected to heat, any primary contraction.

4. A muscle in a state of partial rigor will exhibit a lessened degree of the primary heat contraction.

5. The first contraction is due to the coagulation by heat of the protein present in the muscle fibre during life, the second to changes in the connective tissue elements in the muscle.

6. It thus appears very probable that the different protein substances which can be obtained from a saline extract of muscle are not actually present as such in the living tissue, and the probability exists that at the moment preceding death the muscle fibre contains as its characteristic protein constituent one complex body.



## THE CONDUCTIVITY OF BLOOD IN COAGULATION

BY T. M. WILSON.

*From the Hull Physiological Laboratory, University of Chicago**(Received May 25th, 1907)<sup>1</sup>*

In a paper on 'Some aspects of adsorption phenomena with especial reference to the action of electrolytes and to the ash constituents of proteins,'<sup>2</sup> Dr. W. M. Bayliss makes the statement that he has succeeded in showing that a decrease of conductivity of fowl's blood occurs during the process of clotting, which he attributes to the 'disappearance of ions.' This result, he thinks, is what should be expected from our knowledge of the relation of calcium to clotting. In a single experiment he quotes a decrease of conductivity amounting to no less than  $18\frac{1}{2}$  per cent. I showed some time ago<sup>3</sup> that the conductivity of entire (unclotted) and of defibrinated human blood was practically the same, and that this also was true of plasma, separated from the unclotted blood and of serum from the defibrinated blood. Later on R. T. Frank<sup>4</sup> confirmed my statement, being unable to detect any sensible change of conductivity in clotting.

It may be that a large series of very careful observations might reveal a slight difference, but it is impossible to suppose that a decrease of  $18\frac{1}{2}$  per cent. in the conductivity of blood can be caused by a disappearance of calcium ions in the formation of fibrin, since removal of all the calcium ions in the blood would not produce more than a minute change. It was conceivable that the increased friction due to the jellying of the blood might cause such retardation in the movement of the ions as to account for some part of the difference.

1. Date of Chicago postmark May 14th, 1907.
2. *Bio-Chem. Jour.*, Vol. I, pp. 175-232, 1906.
3. *American Journal of Physiology*, Vol. XIII, p. 139, 1905.
4. *American Journal of Physiology*, Vol. XIV, p. 466.

But this influence could hardly be expected to be great even if, instead of an open felt-work of fibrin threads being formed, the whole plasma set into a homogeneous clot, for it has been shown by Arrhenius<sup>1</sup> and others that the clotting of a gelatin solution containing electrolytes may produce little or no change in its conductivity. And G. N. Stewart<sup>2</sup> determined that the conductivity change is very slight when egg white or solutions of muscle proteids are coagulated by heat.

It seemed most probable, therefore, that the result of Bayliss was due to some error of observation. One error there must certainly have been, that due to sedimentation of blood during the long interval between the first and the last observation. He expressly states that he did not disturb the U tube in which the blood was contained. Some sedimentation must, therefore, have occurred, and it is a familiar fact to those engaged in conductivity measurements on blood that when sedimentation takes place in a column of blood to any extent the electrical conductivity is decreased, owing to the formation of a badly-conducting layer. It is customary to eliminate this error, which otherwise would entirely vitiate such measurements, by shaking or stirring the blood from time to time, or perhaps constantly by means of a special apparatus. The error can be minimised by making the measurements rapidly.

Another possible source of error might have been the allowing of too little time for the blood to acquire the temperature of the bath before the first measurement. Bayliss says that he permitted the temperature to become 'constant,' but does not state how he assured himself that the temperature of the blood was the same as that of the bath. Blood at 40° C. would require some time to cool to 10.2° C., the temperature of his bath.

If at the first observation the blood was still at an average temperature of 15° C., this would correspond to an apparent decrease of conductivity of not less than 10 per cent. Of course, I do not for a moment assert that Bayliss made any such error as this, but

1. *Zeit. f. physik. Chem.*, Bd. III, page 316, 1892.

2. *Studies from the Physiol. Laboratory of Owens College, Manchester*, 1890, p. 124.

the decrease of  $18\frac{1}{2}$  per cent. in the conductivity undoubtedly requires an explanation, and I therefore make the suggestion. With the view of testing the possible magnitude of these errors under similar conditions to those of Bayliss, I made the following experiments. It must be remembered, however, that the rate of sedimentation of different specimens of blood even for two animals of the same species may be notably different.

*Experiment I.*—Rabbit's defibrinated blood was placed in a glass U tube, immersed in a bath whose temperature remained constant at  $7^{\circ}$  C. It was left in the bath for thirty minutes. The U tube had been used in many previous experiments, and it was known that in a much shorter time than this the temperature of the blood would be the same as that of the bath. It was then thoroughly stirred by means of a pipette cooled to  $7^{\circ}$  C., and resistance measurements were taken from time to time. No further stirring of the tube taking place. The interval in minutes from the beginning of the resistance measurements is given in the first column.

Time		Resistance in ohms
0	...	7455
10	...	7520
20	...	7720
30	...	7820
40	...	7880
50	...	7910

Here the resistance increased 455 ohms or 6.1 per cent. in fifty minutes. This is owing to sedimentation, since the stirring up of the blood restores the original resistance. A similar sedimentation will, of course, occur in the entire blood before it clots, and possibly to some extent even after clotting has begun.

*Experiment II.*—Another specimen of rabbit's defibrinated blood at  $35^{\circ}$  C. was put into the U tube, which was then immersed in the bath at  $7^{\circ}$  C. Resistance measurements were begun in five minutes from the time of immersion, the blood not being stirred up throughout the whole series of reading. In the first column intervals from the time of immersion are given in minutes.

Time		Resistance in ohms
5	...	6913
13	...	7297
17	...	7381
32	...	7625
40	...	7703

Here the resistance increased 790 ohms or 11.4 per cent. under the combined influence of sedimentation and fall of temperature. Had the readings been begun earlier, the change would, of course, have been still greater.

*Experiment III.*—About 7 c.c. of blood was obtained through a cannula in the carotid from a rabbit anaesthetised with ether. The cannula was perfectly cleaned, and had been previously boiled. The blood was shed into a perfectly clean small bottle, which was placed in crushed ice. A second sample was similarly caught, and at once defibrinated. After all the fibrin had been removed it was also placed on ice. Two tubes were filled respectively with unclotted and defibrinated blood, and placed side by side in a bath at 7.5° C. By means of keys either of the tubes could at any time be connected with the Wheatstone bridge. The resistances of the two tubes were measured alternately at intervals. The results are given in the table. The resistance capacities of the two tubes were as 1 : 1.55. Defibrinated blood was placed in the tube of greater resistance. The ratio of the resistance of the tube of unclotted blood to that of defibrinated blood was always less than 1 : 1.55 up to the time when the sedimentation of the blood was stopped by clotting. The reason is that the defibrinated blood was somewhat less rich in corpuscles than the unclotted blood owing to the removal by the fibrin from the defibrinated blood of a larger proportion of corpuscles than serum. It should be further added that the figures of the first and perhaps of the second line are inaccurate, owing to the temperature of the blood at the time of immersion being unknown. That of the entire (unclotted) blood was doubtless at first below that of the bath. In the column marked 'calculated resistance' the actually observed resistances of the defibrinated blood are corrected for the difference of time between the measurements for the entire and defibrinated blood. This correction is, of course, only approximate, especially for the first two or three readings.

Entire blood			Defibrinated blood			Ratio of resistance of entire to defibrinated blood
Time	Resistance	Time	Observed Resistance	Time	Calculated Resistance	
0	6600	3	9200	0	9100	1.37
5	6470	9	9400	5	9266	1.43
15	6480	13	9480	15	9490	1.46
25	6490	24	9590	25	9595	1.47
31	6500	32	9600	31	9592	1.47
40	6570	39	9750	40	9751	1.48
49 <sup>1</sup>	6610	47	9760	49	9778	1.47
57	6610	60	9880	57	9853	1.49
113	6616	92	9970	113	10033	1.45
155	6667	—	—	155	10160	1.46
855 <sup>2</sup>	6910	860	12320	855	12345	1.78

1. Clotting occurred at forty-nine minutes.

2. This measurement was made next morning. The increase of resistance is doubtless due to the retraction of the clot in the bend of the tube.



*Experiment IV.*—In another experiment on rabbit's blood (obtained from a cannula in the abdominal aorta) the resistance of the entire blood increased from 4870 ohms after five minutes' immersion in the bath to 6600 ohms (35·5 per cent.) after forty-two minutes' immersion; and to 8170 ohms (67·7 per cent.) after seventy-five minutes' immersion. At eighty minutes clotting began, the resistance being then 8270 ohms. At eighty-six minutes it was 8300 ohms, and at ninety-five minutes also 8300 ohms, the clot being now of such consistence that no further sedimentation occurred. The temperature of the bath varied from 8·1° C. to 8·3° C. The tube containing the blood was the same as that used for the entire blood in Experiment III, the difference in resistance at one and the same temperature being due to the fact that in Experiment IV the blood was much richer in plasma. Doubtless the greater and more rapid sedimentation was connected with this. The time of clotting of the blood was controlled by examining occasionally a specimen in another tube immersed in the same bath, and also (but only after clotting had begun in the control tube) by inserting a platinum wire into one limb of the tube whose resistance was being measured, and removing a minute drop of the blood. This wire was thoroughly cleaned each time by washing with water and alcohol and then heating in the flame.

*Experiment V.*—Hen's blood was obtained through a cannula in the carotid, contact of the blood with the tissues being avoided. A portion was run into a small bottle immersed in crushed ice. Another portion was defibrinated, from which a large quantity of fibrin was formed. The two U tubes were filled respectively with the unclotted and the defibrinated blood and immersed in a bath at 8° C.

Entire blood		Defibrinated blood			Ratio of resistance of entire to defibrinated blood
Time	Resistance	Time	Observed Resistance	Time	
12	4835	7	6890	12	1·41
22	4860	25	7020	23	1·43
30	4882	35	7045	30	1·45
49 <sup>1</sup>	4900	46	7065	49	1·44
55	5100	—	—	—	—

1. Clotting occurred before forty-nine minutes.

*Experiment VI.*—In this experiment hen's blood was also used, but a small portion of muscle was dropped into the U tube to cause coagulation. Temperature of the bath 7° C.

Entire blood		Defibrinated blood				Ratio of resistance of entire to defibrinated blood
Time	Resistance	Time	Observed Resistance	Time	Calculated Resistance	
15	4610	5	6230	15	6360	1'37
20	4680	10	6250	20	6428	1'39
25 <sup>1</sup>	4710	15	6360	25	6496	1'38
35	4715	34	6620	35	6634	1'40
115	4725	109	7130	115	7170	1'51

I have to thank Dr. G. N. Stewart for calling my attention to the possible sources of error in such observations, and for suggesting methods for their elimination.

1. Clotting occurred at twenty-five minutes.

## THE ELECTROLYTES IN PATHOLOGICAL BODY-FLUIDS

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(Received June 13th, 1907)

The chemical study of exudates and transudates has occupied the attention of numerous investigators, mainly during the past forty years. The most interest has been attached to the proteid content, while the other constituents, estimated by Hoppe-Seyler, C. Schmidt, Halliburton and others, have been considered of less importance. In respect of their total quantity these substances are insignificant, but they constitute the bulk of the electrolytes present.

The reflection that some substance or substances might be present in some pathological body-fluids and not in others, consequent on the cause of the effusion, has led to the present research. For instance, when cancer is disseminated over the peritoneum, the cells pour out the products of their metabolism into the peritoneal exudate: in this way certain substances might be present which are absent from other exudates. However, the most important of such substances (in this particular class of case) are likely to be ferments or anti-bodies to the same. The question of the presence of these bodies is not to be dealt with in this account, but I propose to limit myself now to the electrolytes.

Bugarsky and Tangl (1) were the first to make an exhaustive study of the two main groups of electrolytes in the blood-serum of different animals. They used Kohlrausch's method of determining the electro-conductivity of the serum, and from the results of this combined with certain quantitative analysis they made certain deductions. The same method has been employed in the present study of pathological body-fluids. Hamburger (2) has fully criticised the method, but it will be convenient to explain first the various defects of the method, and then show to what extent one may make useful deductions.

I.—The electrolytes of effusions may be classed as (1) chlorides, and (2) electrolytes which are not chlorides (achloride electrolytes). The second group consists of sodium carbonate, sodium sulphate, sodium phosphate, calcium and magnesium phosphates, and the ions into which they may be dissociated. The molecular weight of each of these substances is greater than that of NaCl. One cannot therefore accurately express the second group in terms of NaCl, in representing their molecular or osmotic concentration. The essence of Bugarsky and Tangl's records lies in their calculating all the achlorides in terms of  $\text{Na}_2\text{CO}_3$ . This electrolyte is present in far preponderant quantity (after NaCl) in serum. In effusions the same holds good, though not constantly. After working out the different figures, I have found that for the present purpose it is not a serious error to follow the lead of the quoted authors. At first the calculations were made as follows:—

A given pleural fluid contained 0.062 gram-molecule per litre of NaCl. Its conductivity<sup>1</sup> was 1068, which, for the temperature, is equivalent to that of a solution of NaCl of 0.123 gram-equivalent per litre. The difference, 0.061, gives the concentration of the achlorides in terms of NaCl. The ratio of chlorides to achlorides is then  $0.062 : 0.061 = 1.016$ . This is a simple calculation.

The other method gave these results:—

The chlorides amounted to 0.062 gram-molecule per litre. The degree of dissociation of this (calculated by Arrhenius' formula) is 0.861. The number of molecules plus ions (above formula) is 0.1153. The conductivity of such a strength of NaCl subtracted from that of the fluid gave a conductivity which is possessed by a solution of  $\text{Na}_2\text{CO}_3$  containing 0.062 gram-molecule per litre, with a degree of dissociation of 0.570, and 0.1342 molecules plus ions per litre.

The ratio NaCl :  $\text{Na}_2\text{CO}_3$  is now 1.16.

This calculation is obviously much more prolonged.

1. Throughout this account, 'conductivity' means 'specific conductivity,' and is expressed in terms of  $10^5$ .



The error in these calculations may lie in the values used for the conductivity of NaCl and  $\text{Na}_2\text{CO}_3$  solutions, which have been estimated in *watery* solution, whereas they occur in *albuminous* solution in the body. The degree of dissociation is vastly different. To this we presently return.

The following analysis of pus-serum by Hammarsten (3):—

NaCl	...	...	5.39 %	or,	Chlorides	...	...	5.39 %
$\text{Na}_2\text{SO}_4$	...	...	0.31		Carbonates	...	...	1.13
$\text{Na}_2\text{HPO}_4$	...	...	0.46		Sulphates	...	...	0.31
$\text{Na}_2\text{CO}_3$	...	...	1.13		Total phosphates	...	...	0.94
$\text{Ca}_3(\text{PO}_4)_2$	...	...	0.31					
$\text{Mg}_3(\text{PO}_4)_2$	...	...	0.12					
$\text{PO}_4$ (in excess)	...	...	0.05					

shows the preponderance of carbonates over phosphates and sulphates. It must be admitted that this is not invariable, but it has been found that even if the phosphates be in excess and the mode of expression of achlorides be altered accordingly, the ratio still remains similar. It is obvious that the only way of avoiding errors at all would be by complete chemical analysis. The present method is an attempt to obtain useful results by the simple method of conductivity determination.

II.—*The presence of non-electrolytes.* Those which are present in exudates and transudates are sugar, urea, cholesterin (sometimes), creatin (sometimes), lecithin (ascitic fluid); but their actual amount compared with *albumen* is quite negligible, so that any correction which has to be made for the latter body will cover that needed for the other non-electrolytes.

The retarding influence of albumen on the conductivity of a fluid is 2.5 per cent. for every 1 per cent. of albumen. The formula

$$\text{Corrected conductivity} = \frac{\text{Original conductivity} \times 100}{100 - (\text{percentage of albumen} \times 2.5)}$$

has been used in the results which follow (1).

III.—*The temperature at which the observations are made.* All that need be said on this point is that I have found 18° C. the most convenient to use for the following reasons:—

The low temperature is easily maintained without rapid evaporation of the water in the thermostat; the fluid will not so readily putrefy if the determination should happen to be delayed and the containing vessel remain in the thermostat longer than really necessary; thirdly, the temperature of the fluid comes level with that in the thermostat within ten minutes; and lastly, all the standard observations of conductivity of salt solutions have been made at 18° C., and thus correction formulæ for temperature are avoided.<sup>1</sup>

It may be of use to say that if the temperature be other than 18° C., the value for the conductivity can be corrected to 18° C. by this formula:—

$$k_1 = k_{18} \pm \frac{k_1 \times (t_1 - 18) \times 2.21}{100}$$

IV.—*The estimation of Albumen.* In all the earlier cases (some of which are recorded below) the albumen was estimated with Esbach's instrument, after diluting 1 in 10, in spite of the reputed inaccuracy of the method. The sole reason was the saving of time. Most of the measurements were, however, made by weighing after coagulating the fluid (slightly acid) and drying the coagulum in vacuo over sulphuric acid. The objection was that one had to wait some days before one's analysis was available for the clinician. But it was finally decided to be more important to sacrifice this desire to the need for more accurate figures, since the electrolyte content of fluids is dispensable as an aid to diagnosis, and is of use solely because of the insight into the constitution of these fluids which it affords.

Determination of the weight of albumen by Kjeldahlising was found to be no gain in point of time over simple weighing.

V.—*The estimation of Chlorides.*—With this question is bound up that of the affinity of albumen for Cl. Albumen is well known to combine with Cl if presented to it in the form of acid, but whether it will do so when in combination with Na does not seem to have been discussed. But if albumen be bound up with Cl as a 'solution-

1. For convenience, I have made determination of many strengths of NaCl and Na<sub>2</sub>CO<sub>3</sub> myself, so as to obtain more intermediate values than are obtainable in Kohlrausch's tables. The values for fractions of a degree were calculated from their determinations by Newton's interpolation formula.

aggregate' (4), then when the albumen is coagulated the Cl will presumably go down with it, and not appear in the filtrate which was tested for chlorides by Volhardt's method. More than that, in the coagulum there may be some 'free' chloride entangled which may escape analysis in the same way. The only solution to this difficulty would be to analyse the ash, but this method was cast aside because of necessity for economy in analysing the fluids,<sup>1</sup> and it became necessary to find by experiment whether there be any relation between albumen and Cl which might lead to error in analysis. If there were it would be possible to devise a formula by which the error could be corrected for within reasonable limits.<sup>2</sup>

Solutions of egg-white of various strengths (8, 4, 2 and 1 per cent.) such as would be equivalent to the strength of the albuminous fluids under consideration were made up, and NaCl was added in varying proportions (8, 5, 2, 1 gramme per litre) to each of the albuminous solutions. The fluids were well shaken, allowed to stand overnight, and the chlorides estimated in the usual way. It was then found that if there were only 2 per cent. of albumen, and less than 5 grammes per litre of NaCl, the latter could be recovered from the filtrate after boiling, while if the NaCl amounted to 1 per cent. in such a weak solution, there was distinct loss of chlorine, though only slight even then. With higher strengths of albumen the loss of NaCl was greater, but not proportionately. The error was slight even at the worst.

This shows that in exudates, where albumen may be present to the extent of 8 per cent., the NaCl found volumetrically will be less than that actually present, but not so much less as to cause serious error in the subsequent calculations.

The consideration that even if albumen in a fluid do contain Cl bound up with it, the two together will probably influence the conductivity in the same way as albumen alone, shows once more that we are not introducing any important error by our method.

1. A fairly exhaustive analysis has been made of the proteid constituents and their decomposition-products in these particular fluids as well as of their electrolyte content.

2. Prof. Bayliss' paper in the *Bio-Chem. Jour.*, Vol. I, No. 4, refers to the adsorption of electrolytes by colloids, and here would be an explanation of the error to which I refer.

There is, however, another aspect of the case, and that is with reference to the globulins present in these fluids. The proteid present in pathological body-fluids contains varying ratios of globulins and albumen. The ratio has been called the 'proteid quotient' (Halliburton), which varies from 0.6 to 1.5 in different specimens of fluid. In other words, globulin may exceed albumen in actual amount. Now globulin in the presence of electrolytes behaves as an amphoteric electrolyte, whose particles are not true ions but pseudo-ions (5). The affinity of globulins for salts is the explanation of their precipitation in very dilute media. These points have an important bearing on whether the presence of electrolytes in combination with proteid in a fluid introduces error in the method of calculation. Pauli (6) has made researches on the electrical behaviour of albumen when in combination with Cl, but apparently the alteration produced in albumen by the presence of Cl is not as formidable as that produced in globulin. We have to recognise that the influence of globulin on electro-conductivity is less inhibitory than that of albumen. This will be in favour of achloride electrolytes, for the figures obtained in the tables which follow will represent the conductivity lower than the reality, and the corresponding concentration of achlorides will be raised.



TABLE I

i	ii	iii	iv	v	vi	vii	viii	ix	x	xi
No. of case <sup>1</sup>	Nature of case	Fluid contains chlorine $\frac{\text{g.}}{\text{cc.}}$ in litre	Degree of dissociation ' $\alpha$ '	No. of molecules + ions	Specific conductivity of fluid corrected ( $\times 10^6$ )	Conductivity of iii	Conductivity of chlorides (vi-vii)	No. of molecule ions + ions of a solution of $\text{Na}_2\text{CO}_3$ with conductivity in column viii	Total concentration of electrolytes (v + ix)	
8337	Pleurisy secondary to pneumonia	... '064	'860	'1190	1496	550	946	'1804	'2611	2'23
8338	Tubercular pleurisy ...	... '035	'885	'0639	1169	325	844	'1830	'2469	2'86
7313	Ditto	... '024	'901	'0456	825	205	620	'1334	'1790	2'92
7334	Empyema ...	... '024	'901	'0456	616	205	411	'0866	'1342	1'93
7617	Pleurisy secondary to pneumonia	... '031	'891	'0586	1071	245	826	'1733	'2319	2'95
6677	Tubercular pleurisy ...	... '062	'861	'1153	1068	550	518	'1146	'2299	0'99
6325	Pleurisy secondary to abdominal cancer	... '024	'901	'0456	1096	205	891	'1813	'2269	3'97
6603	Peritoneal cancer (colloid)	... '068	'858	'1263	1183	589	594	'1400	'2663	1'10
6712	" sarcoma ...	... '040	'880	'0752	1188	345	843	'1758	'2510	2'33
6838	" cancer (ovary) ...	... '046	'874	'0863	1165	425	740	'1733	'2596	2'00
A	" (ovary) ...	... '044	'876	'0825	900	495	495	'1066	'1891	1'29
b	" (ovary) ...	... '014	'922	'0269	971	145	825	'1813	'2082	6'74
8344	" (?)	... '104	'837	'1910	1102	855	247	'0516	'2426	0'27
6417	Tuberculous peritonitis ...	... '056	'866	'1055	1171	505	666	'1599	'2654	1'51
7553	Chronic peritonitis (gastric ulcer)	... '043	'877	'0800	1311	425	986	'1875	'2675	2'34
8182	Chronic peritonitis ...	... '078	'852	'1444	1432	720	712	'1663	'3107	1'15
8340	" "	... '042	'878	'0788	965	380	585	'1330	'2118	1'68

1. In Register of Clinical Laboratory.

TABLE II

i	ii	iii	iv	v	vi	vii	viii	ix	x	xi
No. of case	Nature of case	Fluid contains chlorine g. aeq. in litre	Degree of dissociation ' $\alpha$ '	No. of molecules + ions.	Specific conductivity of fluid corrected (x 10 <sup>5</sup> )	Conductivity of iii	Conductivity of achlorides (vi-vii)	pro litre of solution of Na <sub>2</sub> CO <sub>3</sub> with conductivity in column viii	Total concentration of electrolytes (v + ix)	ix — v
6196	Pleural fluid (cardiac) ...	... '107	'837	'1965	1065	875	190	'0424	'2460	0.21
6261	Peritoneal fluid (cardiac) ...	... '100	'838	'1838	1281	840	441	'0990	'3194	0.53
6425	" (renal) ...	... '113	'834	'2035	1326	915	311	'0658	'2847	0.31
5903	" (cirrhotic liver) ...	... '094	'842	'1731	1187	802	385	'0868	'2763	0.50
5710	" (thrombosis of portal vein) ...	... '111	'834	'2035	1283	900	383	'0851	'3047	0.41
7191	" (multiple effusions) ...	... '111	'834	'2035	1215	900	315	'0658	'2848	0.32
7381	" (cirrhosis of liver) ...	... '113	'834	'2035	993	915	78	'0140	'2224	0.59
7559	" ( " ) ...	... '088	'845	'1623	974	780	194	'0426	'2121	0.26

TABLE III

i	ii	iii	iv	v	vi	vii	viii	ix	x	xi
No. of case	Nature of case	Fluid contains chlorine g. aeq. in litre	Degree of dissociation ' $\alpha$ '	No. of molecules + ions.	Specific conductivity of fluid corrected (x 10 <sup>5</sup> )	Conductivity of iii	Conductivity of achlorides (vi-vii)	pro litre of solution of Na <sub>2</sub> CO <sub>3</sub> with conductivity in column viii	Total concentration of electrolytes (v + ix)	ix — v
7196	Fluid from leg (cardiac) ...	... '128	'827	'2338	1420	1100	320	'0658	'2078	0.28
	" (cardiac) ...	... '124	'837	'1910	893	855	38	'0990	'2900	0.51
6668	Cerebro-spinal fluid (tumour) ...	... '161	'814	'2920	1294	1260	34	'0990	'2284	0.33
6658	" (syphilis) ...	... '157	'814	'2847	1259	1240	19	'0990	'2249	0.34
6643	Ovarian cyst ...	... '001	'970	'0019	1042	20	1022	'1442	'2484	6.01
6151	" ...	... '040	'880	'0752	1075	360	715	'1680	'2755	2.23
7618	" ...	... '145	'821	'2640	1080	1080	0	0	'2640	0.00
6178	" (? peritoneal) ...	... '001	'970	'0019	1104	20	1084	'1442	'2546	6.01

Study of the headings in the tables of results will render the meaning of the figures obvious, with the exception of those in the last column, which are obtained by dividing the osmotic concentration of the achlorides by that of the chlorides. Table I contains those fluids which may be classed as exudates (the result of definite inflammatory processes in the serous membranes); the second table shows the transudatory fluids (back-pressure effects, or renal dropsical fluids), and the third table shows the values for a few other fluids taken for comparison<sup>1</sup> with those of I and II. The last column is the one which is of chief interest. It reveals a very striking uniformity in the different classes of fluid. The fraction is seen to be greater than one in nearly all exudates, and to be invariably less than one in all fluids which are not associated with active inflammatory processes in the serous membranes. This shows that the osmotic concentration of the achlorides is preponderant in the former case. An examination of the first column will, however, show the almost constant deficiency of exudates in chlorides as shown by quantitative analysis: transudations contain a very considerable proportion of NaCl. The high chloride content of transudates is naturally associated with a low achloride content. The fact that the conductivity of the two sets of fluids is practically constant is of interest as showing that even in the presence of much albumen the conductivity will be the same as that of a fluid containing less albumen because of difference in electrolyte content. This indicates that in the exudates the low content of NaCl (which is more completely dissociated) counterbalances the retarding influence of the albumen on the conductivity.

In Table III the most striking feature is the usual preponderance of achlorides in ovarian cyst fluids. In fluid taken from the leg in cases of cardiac dropsy the achlorides are deficient, as one might expect.

To return again to Table I. The conductivity of the fluid in three cases of peritoneal malignant disease (6603, 6712, and 6838) will be seen to be practically the same, and these three cases are very

1. A full account of these is reserved: many other specimens have been examined.

like No. 6417, one of tuberculous peritonitis, as regards their conductivity. As regards degree of dissociation these fluids are practically identical, which shows that one cannot hope to diagnose the nature of the fluid by mere determination of the electro-conductivity. On the other hand, one might reasonably expect the products of metabolism in the case of cancer to be different from those of tuberculous masses, and therefore expect a difference in the constitution of the two classes of fluid. It becomes evident that, if this deduction be reasonable, the difference must be one concerned with non-electrolytes.

The next question is as to the reason for the different ratios ( $C_{NaCl} : C_{achlor.}$ ) in the different fluids. The reason must be sought for in a difference in permeability of serous membranes to various ions under different circumstances. As regards inorganic salts the permeability seems to be only limited by the rate of migration of the ions of which the salts are composed. Cl passes through most readily, then  $NO_3$ , and  $SO_4$ ; Na passes more quickly than Mg (Höber 7). Another part of the explanation lies in the power which albumen has of attracting water to itself. Starling showed the effect of having a strong solution of albumen on one side of a serous membrane, and a weak solution containing salts on the other. Moreover, the laws of osmosis are different for watery solutions to what they are for albuminous solutions, and in the case of peritoneal fluids we have to deal with fluids which are frequently hypertonic. If there be as much albumen on one side as there is on the other there will be no movement of water from one side to the other.

It is easy to explain the characters of transudates. In renal disease there is decided inadequacy on the part of the kidney to get rid of NaCl, which consequently becomes more concentrated in the tissues and lymph spaces, &c. Water is necessary for its solution, and hence the effusions. This accounts for the excess of chlorides in these fluids.

Lastly, attention must be drawn to three of the cases. In one case (of peritoneal cancer) (8344) the ratio (last column) is less than one. In the second case (6677) the ratio is again less than one. The explanation for the former cannot be found at present, and it must



be left as an exception to the general rule. In the other case it becomes a question whether the fluid was really of inflammatory origin. The case was one of 'pleural effusion,' cause not stated. The third case is in Table II, No. 7191. There was thickening of all the serous membranes with effusions into them :—'polyorrhomenitis.' In the light of the facts made out one would say that in this case the fluid passes out for similar reasons to that which holds in the case of transudates in general. The nature of the disease is not yet fully established.

I am indebted to Prof. Moore for the suggestion that the characters of exudates depend on the cellular elements which occur in them. The presence, in cells, of more phosphates than chlorides, would explain the preponderance of achlorides in the fluid. This consideration would give an explanation for the finding in No. 7191, for with thickened peritoneum there would be very few cellular elements present.

The conclusions are :—

1. The method adopted is a convenient one, but requires cautious interpretation.
2. Transudates invariably contain an excess of NaCl over achloride electrolytes.
3. Exudates contain a relatively smaller number of chlorides. Whether they contain an absolutely greater number of achloride molecules per litre is not beyond dispute.
4. Ovarian cyst fluids usually contain more achlorides than chlorides.
5. It is substances other than electrolytes which are mainly of importance in the study of exudates.

In conclusion, I should like to express my indebtedness to many of the Honorary Staff of this Hospital for permission to record these observations on their cases.

## LITERATURE

1. Bugarsky and Tangl, *Pflüger's Archiv.*, 72, 1898.
2. Ionenlehre, I, 500 et seq.
3. Physiological Chemistry. 4th ed.
4. See Moore and Roaf, *Bio-Chemical Jour.*, Vol. II, No. 1.
5. Hardy, *Journ. of Physiology*, Vol. XXXIII, 251.
6. *Naturwissenschaftliche Rundschau*, 1906.
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## ABNORMAL FAT ASSIMILATION ASSOCIATED WITH SOME DISEASES OF THE INTESTINE

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The difficulties surrounding the study of the absorption of fat and the different views held thereon depend largely upon the limitation of knowledge regarding the various fats concerned, and the difficulties of the chemical technique connected therewith. Except in such conditions as the failure of the fat-digesting secretions to reach the intestinal tract associated with obstruction to the flow of bile and pancreatic juice, little is known of any abnormality of fat digestion. The present views of the digestion of fats is stated by B. Moore (1) as follows :—

‘It is now generally accepted that the neutral fats of the foods are completely split up in the course of intestinal digestion by the lipase of the pancreatic juice into free fatty acids and glycerine, and that the fatty acids are then rendered soluble by the bile salts and lecithin, either in the form of free fatty acids or as alkaline soaps, so that all the constituents formed in fat digestion are finally taken up in soluble form.’

In the same contribution this author points out that from solutions containing soaps and glycerine the living cell is capable of inducing the synthesis of neutral fat, and thus rendering the soaps capable of absorption.

The clinical and chemical observations recorded in this contribution tend to show that there are conditions in the intestines in which

certain soaps or other insoluble compounds of fatty acids are not absorbed by the intestinal mucous membrane, and that with the presence of these conditions certain diseases of the alimentary tract are related. The analyses here given show that true intestinal sand, appendix concretions, and some bodies formed in the intestines are largely formed of fats and soaps. The fats and soaps present are compounds of saturated fatty acids. The constitution of these bodies and their relation to each other in chemical constitution throws, in my opinion, much light upon the etiology of appendicitis and mucous colitis, and possibly upon the underlying factors in certain diseases accompanied by colic.

Investigations of other foreign bodies different from the above occurring in the intestine, such as concretions formed by inspissated faeces, by inorganic salts, gall stones, and *false* intestinal sands, have been made, but their chemical constitution shows that they have no relation to the pathological processes accompanying fat digestion.

In the following analyses determinations have been made of total ethereal extractive, free fatty acid and combined fatty acid, soaps insoluble in ether, iodine values of the fatty acids showing percentages of saturated and unsaturated fatty acid present (Hubl and Köttstorfer methods, non-saponifiable matter, cholesterine, etc.), and the bases present in combination with fatty acids and in organic combinations.

In the present state of our knowledge a systematic classification of fat is not possible. In the investigation of fats in faeces it is usual to determine the melting points, and to use these as expressions of their different natures. A more correct classification for practical purposes, as the results of these observations show, appears to be obtained by using their iodine values. This value expresses the amount of iodine which 100 grammes of fat are able to take up and combine with, and divides the fatty acids into two main groups, the saturated and the unsaturated. Of the saturated fatty acids palmitic and stearic acids, and of the unsaturated fatty acids oleic acid, occur in the animal fats.



## INTESTINAL LITHIASIS

Cases in which intestinal sand has been passed in the stools have been recorded from time to time, the most noteworthy being the one recorded by Duckworth and Garrod (2). In this as in others the amount of organic and inorganic material, with the nature of the latter, has been recorded. I have only been able to find one observation which calls attention to the presence of fat in the material, Roesser (3) reporting that 10 per cent. of his specimen contained fatty substance soluble in ether.

The sand is found as a 'gravel' adhering to the bottom of the vessel from which the faeces have been emptied. It varies in colour from grey to brown, and is often mixed with small quantities of faeces which tend to hide its appearance as an abnormal constituent. When the specimens are washed and dried they in all cases show the characteristic appearance of the sand. This looks much like ordinary sand, but has not the same gritty feeling, but rather feels like soap, and in well-marked cases I have observed fair-sized particles appearing and feeling like ordinary soap. My attention was first drawn to this material by Professor Vaughan Harley, at whose request I examined it in his laboratory.

The first specimen examined was intimately mixed with numerous small green leaves, and microscopically show vegetable fibres in abundance. False intestinal sand consists of the remains of undigested vegetable food, and thus this specimen suggested some possible relation between the two forms. In two later cases of the true sand a few vegetable fibres were found, mixed with the material.

The true sand was found to be insoluble in water. On heating with Millon's reagent some of the particles became faint red in colour.

By analysis it was found to contain—

					Per cent.
Organic material and water	...	...	...	...	55·6
Inorganic material	...	...	...	...	44·4

The ash after combustion contained—

	Per cent.
CaO ... ..	65·65
Fe <sub>2</sub> O <sub>3</sub> ... ..	6·5
MgO ... ..	0·15
P <sub>2</sub> O <sub>5</sub> ... ..	23·1
CO <sub>2</sub> ... ..	3·3
SO <sub>4</sub> ... ..	1·3
Cl ... ..	Trace
Total fatty acids in specimen ... ..	18 per cent.

Of the total calcium present, only 34·1 per cent. is present as calcium phosphate. The remaining 31·5 per cent., as is seen from the above table, has no inorganic acid with which it could be combined.

An investigation was therefore made to find the combination in which this calcium existed. The material on being burnt showed a smoky white flame, which suggested the presence of fat. The original material was accordingly treated with ether, and 10 per cent. of its total weight was found to be soluble.

This ethereal extract was found to contain :—

22·5 per cent. free fatty acid	} estimated as oleic acid
13·4 „ combined fatty acid	

The iodine value of these fats was found to be 18 per cent.

After all the fats soluble in ether had been removed the material was treated with hydrochloric acid, and again extracted with ether, when a further 8 per cent. was found to be soluble. This was found to be free fatty acid, the hydrochloric acid having therefore split up the calcium soaps and liberated their fatty acids.

The iodine value of these fatty acids was found to be 13·3 per cent.

The above values show that the fats present in the material were of the saturated type (palmitic or stearic, or both).

During the past year, in the wards of the Royal Infirmary, Liverpool, I have seen several specimens of faeces containing particles resembling sand. I have been able to collect seven well-marked specimens, and in these I have found the same constitution as that noted above, all showing presence of fat soluble in ether and soaps

insoluble in ether, all the fatty acids from these being of the saturated type. To obtain the material from well-formed stools they were allowed to stand under a slowly-running tap to disintegrate them, and then carefully emptied, the sand remaining at the bottom of the vessel.

The nature of the cases is shown in the following brief summary. Cases I and II will be referred to in greater detail later :—

*Case I.*—A female, aged twenty-two, under the care of Sir James Barr. The patient's appendix had been removed one year previous to admission to hospital on account of repeated attacks of abdominal pain. The pain recurred one month after operation. On admission she was suffering from abdominal pain with much mucus in the stools.

*Case II.*—Under Mr. Paul's care. A male, suffering from symptoms suggesting the presence of gall stones. The patient had colic.

*Case III.*—A female, aged sixteen, under the care of Sir James Barr. The patient had a large ovarian cyst. On admission she was suffering from abdominal pain, constipation, and vomiting. The vomiting had no relation to food. Intestinal sand was found in the stools both before and after the operation for removal of the cyst.

*Case IV.*—Sir James Barr's case. A female, aged thirty-four, suffering from colic. She was markedly constipated. Purgatives relieved the pain after the passing of faeces containing intestinal sand. The appendix vermiformis was felt to be thickened.

*Case V.*—Sir James Barr's case. A female suffering from thrombosis of the femoral vein following parturition. The patient was very ill and the leg much swollen and very painful.

*Case VI.*—A male, under the care of Dr. Bradshaw, suffering from ulcerative colitis, with pus and blood in the stools.

*Case VII.*—A female, under the care of Mr. Paul, suffering from malignant disease of the caecum. The caecum was removed. Intestinal sand was found both before and after operation.

Harley and Goodbody (4) state that the sand is not uncommonly found in the stools if looked for. On many occasions, in addition to the seven typical cases mentioned above, I have been able to find small quantities of similar material by placing the faeces in a bag with an open top, made of several layers of muslin, and allowing it to hang in running water until most of the faeces had been washed away. The material when present in any quantity was found in cases where there was abdominal pain of a colicky nature, and it may hence be suggested that the material acted as a chemical irritant,

giving rise to painful contractions of the intestine to eliminate it. The richness of these intestinal sands in calcium, iron, and phosphoric acid, and also in fatty constituents which are all usually described as constituents of the secretion of the intestinal mucous membrane, point to their originating in the intestine as a result of some excess in secretion or defect of absorption.

Duckworth and Garrod investigated the pigments in their case, and from the relatively large amount of urobilin and the small amount of unaltered bile pigment present, believed it to be formed in the upper part of the colon.

#### APPENDICITIS

Concretions occurring in the appendix when dried cut quite easily; they appear almost always to be white, and the cut surface has a smooth appearance like soap. This is quite different from the section of a faecal concretion, which is deeply pigmented and has a gritty feeling to the touch.

Analyses of these concretions show them to be very similar in constitution as regards fat to the specimens of intestinal sand, both containing fats soluble in ether and soaps insoluble in ether.

The following tables show the nature of the fats contained in three concretions obtained from the appendix:—

##### FAT SOLUBLE IN ETHER

	Amount	Combined fatty acid	Iodine value
Specimen A ...	14.97 per cent.	73 per cent.	4 per cent.
„ B ...	16.2 „	all	16 „
„ C ...	7.1 „	all	18 „

##### FATTY ACIDS FROM SOAPS INSOLUBLE IN ETHER

	Amount	Iodine value
Specimen A ...	15.6 per cent.	4 per cent.
„ B ...	2.1 „	low
„ C ...	1.2 „	low

The amount of calcium in each concretion was found to be—

A, 3.3 per cent.      B, 3.6 per cent.      C, 3.2 per cent.



A faecal concretion from the intestine examined at the same time for comparison was found to contain *only* '68 per cent. of calcium.

The extraction of fat from several faecal concretions was undertaken for comparison, but the amount and nature could not accurately be determined owing to the amount of pigment always taken up by the ether interfering with the reactions—another point of difference between an appendix concretion and one formed of inspissated faeces.

Further facts which point to the fact that appendix concretions are not, as they are almost invariably called, faecal concretions are the following :—

Appendix concretions are usually of sufficient size to markedly dilate the terminal portion of the appendix, and to do this it would presuppose an abnormal amount of force acting from the caecum to so distend the narrow lumen, a condition of affairs impossible to understand with the lumen of the caecum so large to carry away the faeces acted on by such a force did it conceivably exist. The appendix concretions are invariably concentrically laminated, proving conclusively that they are formed by successive depositions on all sides, the terminal as well as the proximal, and that they are formed *in situ*.

That the appendix has a secretion which, under abnormal conditions, could form these concretions, is to be seen in any appendix in the post-mortem room. Sir William Macewen has demonstrated the presence of such a secretion during life. Schmidt (5) states that the intestinal mucous membranes excrete inorganic salts, such as those of iron, calcium, and phosphoric acids, and also fatty substances. Hermann (6) has shown that a loop of intestine separated from the intestinal tract, sewn up in the form of a ring, and restored to the abdomen, soon becomes filled with a mass resembling faeces.

It is probable that the faecal-like masses found in the appendix are formed in a similar manner; this would explain their containing fat and calcium in such large quantities.

In a case in the Royal Southern Hospital, under the care of Mr. Newbolt, I was able to collect a glairy mucoid-looking material which was being excreted by a loop of intestine shut off from the

rest of the alimentary tract and discharging on the surface. This contained 3·8 per cent. of fatty substance which contained saturated fatty acids (iodine value 12·2 per cent.). This shows that the fatty substances excreted by the intestinal mucous membranes are of the saturated type.

The above facts are evidence to show that the concretions in the appendix containing insoluble calcium soaps of saturated fatty acids are formed by the secretion of the appendix itself.

It is generally agreed that these concretions, when they occur, play a definite rôle in the pathogenesis of appendicitis. Of a case observed, Nothnagel (7) states :—

‘The appendix after removal was opened while still warm, and while the muscular fibres were still contracting. The mucous membranes were pale and perfectly normal in appearance except at the distal extremity in the immediate vicinity of a hard rounded calculus, the size of a cherry stone, where it was injected, and light red in colour. An observation of this kind conclusively shows, to my mind, that the localised catarrh, which was unquestionably due to the presence of the faecal concretion, would have ultimately led to other and probably grave results.’

Though the majority of cases occur without the formation of a concretion, it may be suggested that the same pathological processes which allow of their formation may have a deleterious effect even before a concretion is formed. The appendix normally tends to become obliterated as the result of age. Zuckerkandl (8) shows this to be the result of involution changes :—

‘The mucous membrane undergoes atrophy, the glandular structures desquamate, and the opposing layers of the inner surface of the organ grow together. At the same time, or occasionally before this time, the submucous coat undergoes thickening, and *fat* accumulates at this part of the wall. The muscular coat does not necessarily show any changes, but may also reveal an accumulation of fat.’

The lumen of the appendix is so small that the slightest swelling of its wall leads to its obliteration. I have cut open several appendices

not containing concretions immediately after their removal by operation and placed them in osmic acid. The walls stained black, but on the surface of the lumen there remained a layer which did not stain, this layer being the secretion adherent to the walls. This is significant, as the saturated fatty acids—palmitic and stearic acids—do not stain with osmic acid, whereas unsaturated fatty acids such as oleic acid do. I would therefore suggest that these fat compounds secreted or excreted by the tubular glands may under abnormal conditions block the lumen of the gland, and so render them liable to be more easily infected by micro-organisms than they would be if carrying out their normal functions.

The close relationship between appendicitis and colitis has been frequently suggested. Harley and Goodbody (9), in discussing the etiology of mucous colitis, do not admit that it is generally consequent on appendicitis. A great number of cases undoubtedly follow appendicitis, but this fact does not necessarily imply that the appendicitis is responsible for the condition. The underlying pathological condition which caused the appendicitis is probably the same as that which caused the colitis.

Intestinal lithiasis is, according to Nothnagel, usually associated with mucous colitis, and may be prolonged over many years.

He also states that it has been regarded as due to a lithogenic catarrh of the bowel, and might be compared to the process which gives rise to the formation of concretions in the vermiform appendix.

In relation to the above views, Case I, mentioned above, is of interest.

The appendix was removed in November, 1905, on account of repeated attacks of abdominal pain. The patient stated that one month afterwards the same pain recurred, and continued intermittently until December, 1906, when she was admitted to Sir James Barr's wards. Colic was present at this period, with attacks of nausea and vomiting. The vomiting was unrelated to food. The patient was of a neurotic temperament. In the stools were found much mucus and quantities of intestinal sand. The sand was not immediately obvious, and was gathered by the methods mentioned

above. It was analysed and shown to be true intestinal sand. After some weeks' treatment the symptoms and stools were much the same as on admission. With the view of relieving the symptoms the patient was placed on a diet containing much unsaturated fat (olive and sardine oils, both of which have very high iodine values). After a week on this diet the amount of sand and mucus in the stools was very much diminished. On the patient resuming a diet containing butter fat and meat fats (saturated), the sand and mucus increased very definitely in quantity. The stools were carefully collected under these conditions, and are being subjected to special examination for separation and determination of the different saturated and unsaturated fatty acids. After three months' treatment with olive oil, the pain is much less, and there is very little sand or mucus in the stools.

The fact that unsaturated fats might be of value was suggested by obvious theoretical considerations, the formation of more soluble and more easily split-up soaps. This is borne out in a case related to me by Dr. Thomas Clarke, who, many years ago, had a patient suffering from abdominal symptoms, with which was associated intestinal lithiasis. Treatment for a very considerable time failed to relieve the patient until she was placed on a diet containing olive and salad oils, when the symptoms and sand both disappeared in a few weeks.

French authors (Dieulafoy, Matthieu, Talamon, Reclus, and others) attach special importance to a peculiar form of catarrh as being the cause of the formation of intestinal sand, a catarrh which they consider to stand in close relationship to enteritis membranacea or also to gout, and which, according to some, is also the cause of appendicitis. Dieulafoy (10) has called attention to the number of patients suffering from muco-membranous typhlo-colitis who are operated on for an appendicitis which they have not got. He described thirteen cases, all suffering from abdominal pain. It has frequently occurred that patients have been operated on for appendicitis when they have had entero-colitis, and Dieulafoy has observed the painful crises to recur at the same spot and with the same intensity



after the operation. Bottentuit (II) has similarly collected twenty-two cases who have suffered from muco-membranous typhlo-colitis, or intestinal lithiasis, who have been operated on for a non-existent appendicitis.

It is therefore possible that the observations recorded above may afford a solution to the suggestions which have been made as to the relations between appendicitis and mucous colitis on the one hand, and mucous colitis and intestinal lithiasis on the other, and go still further to suggest a possible relation between the three diseases, they having in common some abnormality in the conduction of the processes associated with the digestion and absorption of fat, and the production of calcium salts of saturated fatty acids.

#### FAT CONCRETIONS IN THE INTESTINE

That these are not the only conditions in which pathological fat processes are associated with disease is seen from the following cases :—

From a patient suffering from acute intestinal obstruction Mr. Paul removed a mass which was causing the obstruction. It was situated in the small intestine in the upper part of the jejunum. Recognizing the unusual character of the mass, Mr. Paul kindly sent it to me to examine. It was about the size of a small Tangerine orange. In the centre was a raisin, around which was a bright yellow pultaceous mass held together by stroma of vegetable material. The exterior was stained by faecal material.

The nature of the fat was found to be—

Amount	Combined fatty acid	Iodine value
34·1 per cent.	74 per cent.	18 per cent.

The unsaponifiable material was isolated, and proved to be cholesterine. That this was not a concretion such as is sometimes formed by the administration of large quantities of olive oil was proved by the nature of the fat.

In another case of Mr. Paul's, a male, the symptoms suggested the presence of gall stones. Intestinal sand was found in the stools.

When operated upon there were found in the bile passages and neighbouring portions of the intestines numerous very small brownish-red granules, but no stones. An analysis of these granules showed that they contained fat in quantity.

This specimen is of interest in relation to the site of formation of intestinal sand, as the presence of the sand in the stools with the numerous fat granules in the duodenum suggest some relation between the two by their synchronous appearance in the same patient.

Duckworth and Garrod (12), in their specimen of intestinal sand, found very little unaltered bile pigment, showing that their specimen was probably formed lower down in the intestine.

The fact that the fatty acids present in all the above conditions are saturated—such as palmitic and stearic—is of high interest, for it is well known that these fats, when given alone, are only absorbed in very small quantities, from 86 to 91 per cent. remaining as residue in the faeces, whereas, when given mixed with unsaturated fatty acids, such as oleic, they are absorbed to a higher degree. Olive oil, when given alone, is almost all absorbed, only 2 per cent. remaining in the faeces.

Equally interesting, from the point of view of the value of the various fats in human food, is the fact that human milk contains a very high proportion of unsaturated fatty acids compared with the milk of the cow. The fact that unsaturated fatty acids aid the absorption of the saturated suggests that in the above conditions, where insoluble compounds of saturated fatty acids occur, the administration of olive oil might be of value in aiding the absorption of these deleterious products.

Observations which are being made tend to show that the nature of the fats in the diet have some effect in producing the conditions mentioned in this paper.

My thanks are due to Professor Vaughan Harley, in whose laboratory the work was commenced, and to Mr. Thelwall Thomas for many valuable specimens. To the surgeons and physicians of the Royal Infirmary, Liverpool, I am indebted for the use of their cases,

for specimens, and for the time to conduct these researches. The work was conducted in the Bio-Chemical Laboratory of the University, under the direction of Professor Benjamin Moore. My thanks are due to him for his valuable advice, and to my colleagues in the laboratory for much kind assistance.

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- (2) *Med. Chir. Transactions*, Vol. LXXXIV, 1901, p. 389.
- (3) Smith and Strasburger, *Die Faeces des Menschen*, 1905, p. 248.
- (4) Harley and Goodbody, *The Chemical Investigation of Gastric and Intestinal Diseases*, p. 246.
- (5) Van Noorden, *Metabolism and Practical Medicine*, Vol. II, p. 211.
- (6) Schäfer, *Text-book of Physiology*, Vol. I, p. 473.
- (7) Nothnagel, *Diseases of Intestines and Peritoneum*, 1905, p. 382.
- (8) *Ibid.*
- (9) Harley and Goodbody, *loc. cit.*, p. 211.
- (10) *British Medical Journal*, 1906, June 6, p. 1373.
- (11) *Ibid.*
- (12) *Med. Chir. Trans.*, *loc. cit.*

## A NOTE ON THE REDUCTION OF ALKALINE COPPER SOLUTIONS BY SUGARS

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Anyone who has used Fehling's solution for the detection of dextrose in the urine is familiar with the different coloured precipitates obtained under various conditions, as products of the reduction of this reagent. Thus the precipitate obtained may be red, yellow or even green. The commonly accepted explanation of the different appearance of these precipitates has been that the red compound consists of cuprous oxide,  $\text{Cu}_2\text{O}$ , while the other precipitates represent hydrated forms of this compound. In a recent paper Maclean,<sup>1</sup> on the contrary, maintains that in each case the precipitate obtained is the cuprous oxide, the colour of the precipitate being dependent merely upon the state of division in which this substance exists. The red precipitate is the least finely divided, the yellow one consists of smaller particles, while the green one is in a *still finer state* of division. The evidence on which Maclean bases his explanation is briefly as follows:—  
(1) Microscopic examination of the different precipitates shows coarsely granular particles in the case of the red substance, while the other precipitates consist of smaller particles. (2) The green and yellow precipitates settle with much greater difficulty than does the red one. (3) If the red suboxide be dried and finely ground in a mortar it becomes somewhat yellowish in appearance.

Interesting as are these observations, the fact that the yellow and green precipitates are made up of smaller particles than the red

1. Maclean, *This Journal*, Vol. II, p. 156, 1907.



one, is not convincing evidence that the same compound is involved in each case. Cupric oxide separates from a suspension in water in large masses much more rapidly than does the hydroxide; yet this is no evidence that they are the same chemical substances.

In Maclean's work comparisons are made of the colour of the precipitate obtained in the case of urine containing 0.4 per cent. dextrose and that yielded by pure sugar solutions of equal concentration. The solution of the sugar in urine gave yellow or green precipitates while the pure solution in every case yielded a red product as a result of the reduction. Maclean points out that the substance determining this difference in the character of the precipitate is creatinin, and holds that this causes the precipitation of the suboxide in a finer state of division than occurs when creatinin is absent.

If one is unwilling to admit the validity of Maclean's explanation, the question of the relative dehydrating action of the various solutions used is at once raised. Since the action of creatinin in modifying the colour of the precipitates is quite obscure, it may be well to ask to what extent variations in the dehydrating power of the solutions used may effect the character of the reduction product obtained in pure sugar solutions. Some light may be thrown upon this question by using pure solutions of known different dehydrating powers, and then noting the colour of the precipitates obtained under these conditions. If Maclean's explanation is correct, there should be little if any difference found here; or if a difference be found, we should hardly expect, in view of his explanation, that the results would be exactly parallel with those produced by the urine.

In a recent paper, the use of copper solution in which the alkalinity is secured by means of carbonate instead of hydroxide was suggested for the detection of sugars.<sup>1</sup> In that same paper it was further pointed out that the yellow or green precipitates are obtained

1. Benedict, *Journ. of Biolog. Chem.*, Vol. III, p. 101, 1907. The carbonate reagent mentioned is made up from two solutions prepared according to the following formulae:

Solution A: Crystallized copper sulphate, 69.3 grams.; distilled water to 1,000 c.c.

Solution B: Pure Rochelle salt, 346 grams.; anhydrous sodium carbonate, 200 grams.; distilled water to 1,000 c.c.

For use these solutions are freshly mixed in equal proportions and the resultant mixture diluted with an equal volume of distilled water.

with the carbonate reagent much more *frequently than with* Fehling's solution, and the explanation was suggested that this is due to the much stronger dehydrating action of the hydroxide solution than that of the carbonate. The following further experiments throw light upon the problem. When one half volume of one per cent. pure dextrose solution is added to the carbonate reagent and the mixture warmed to boiling a distinctly green precipitate is first obtained, which, upon further heating, becomes bright yellow. This precipitate will then slowly become reddish in tinge, and may become distinctly red. By substituting Fehling's solution for the carbonate reagent a bright red precipitate is obtained almost at once. In these instances the less strongly dehydrating reagent shows changes in colour of the precipitate as might be explained by assuming that the first products are the hydrated oxides which then gradually lose water. A much stronger dehydrating agent would not permit these changes to be seen so distinctly if at all, hence the much more rapid formation of the red suboxide with Fehling's reagent. The concentration of sugar is the same in both cases, and no disturbing factor such as creatinin is present. The following experiment is also of interest in this connection. If to a few cubic centimetres of the carbonate reagent is added one half to one volume of 0.1 per cent. dextrose solution and the mixture heated to boiling, the *precipitate formed* will remain distinctly greenish-yellow for some minutes. Just after the solution has reached vigorous boiling it is divided into two equal portions. To one of these is added an equal volume of distilled water and to the other an equal volume of ten per cent. potassium hydroxide solution. Both are now heated to vigorous boiling. The one containing the excess of hydroxide rapidly becomes bright red in colour, while the other remains yellow for some minutes. The potassium hydroxide serves in this case to convert the yellow precipitate into the red substance. When it is remembered that potassium hydroxide solution of this strength is a strong dehydrating agent the probable explanation appears to be that the red compound represents a dehydrated form of the yellow one. It might be assumed that the alkali acts here simply as an electrolyte to cause the smaller

particles, possibly in a semi-colloidal state, to unite to form larger masses. But if saturated sodium chloride be used in place of the hydroxide, the change in colour of the precipitate from yellowish-green to red does not occur.<sup>1</sup> It appears more likely that the excess of hydroxide exerts a dehydrating action than to assume that by purely physical action the solid particles already out of solution are brought together into larger masses.

There is no apparent reason for assuming a different explanation for the colour phenomena in the urine reaction and *those obtained with* pure sugar solutions. In view of the comparable reactions obtained with the latter solutions it seems premature to give up entirely current conceptions regarding the nature of the coloured precipitates formed by the reduction of alkaline copper solution by means of sugars.

1. For suggesting the possible objection that the hydroxide might act here simply as an electrolyte and the use of sodium chloride solution as a control test, I am indebted to Professor L. B. Mendel.

# ON THE EFFECT OF NARCOTIC AGENTS IN THE DETACHMENT OF ELECTROLYTES FROM CELL PROTEINS<sup>1</sup>

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Moore and Roaf, as the result of experiments on the comparative vapour pressure of chloroform when dissolved in water, saline, serum and haemoglobin solution, showed that serum and haemoglobin solution, for the same strength of chloroform, always possessed a lower vapour pressure of chloroform than did saline or distilled water.<sup>2</sup>

In a later paper the same authors showed that in lowering the vapour pressure of chloroform, emulsions of tissue cells had a greater effect than emulsions of the ethereal extractives from the same source and of the same strength as in the original emulsion of tissue cells.

From these results it was suggested that chloroform formed a loose chemical compound or physical aggregate with proteins and that anaesthesia results from paralysis of the activities of the protoplasm by the formation of such aggregates. Further, from the greater solubility of other anaesthetics in serum than in water, it is probable that they act in a similar manner.<sup>3</sup>

Observations on the electrical conductivity of saline and serum before and after the addition of chloroform showed that the same

1. The expenses of this research have been defrayed from a grant given by the Government Grant Committee of the Royal Society.

2. *Proc. Roy. Soc.*, Vol. LXXIII, p. 382, 1904, and *Thompson Yates and Johnston Laboratories Reports*, Vol. VI, Part I, p. 151, 1905.

3. *Proc. Roy. Soc.*, B. Vol. LXXVII, p. 86, 1905.



percentage of chloroform caused less diminution of conductivity when added to serum than when added to saline, and that under certain conditions the conductivity, instead of being diminished, was actually increased by the addition of chloroform to serum.<sup>1</sup>

It was suggested that this increase of conductivity was due to the detachment of electrolytes from the proteins under the influence of the chloroform.

Alcock, using frog's nerve, found that chloroform and ether affected the injury current. If the anaesthetic was applied to the whole nerve the injury current was abolished. When it was applied to the cut end the current was increased, but when applied to the longitudinal surface the anaesthetic diminished the electrical potential. In uncut nerves a current was caused by local application of the anaesthetic, and the direction of the current was such that the anaesthetised portion became negative to the rest of the nerve.<sup>2</sup>

These electromotive effects could be explained by the same process that Macdonald showed to take place in an injured portion of nerve. Dead or dying portions of nerve have the electrolytes liberated from their 'masked' condition and thus the injury current is caused by the diffusion of the ions from a point of higher to that of lower concentration.<sup>3</sup>

Alcock states that the electrolytes are not liberated by the anaesthetic as the electrical conductivity was not increased after the nerve was anaesthetised,<sup>4</sup> but Waller found a diminution of resistance when a nerve was chloroformed.<sup>5</sup>

In a second paper Alcock showed that the resting current of frog's skin was abolished by the application of chloroform to the outer surface, but it was unaffected when the anaesthetic was applied to the inner surface. He also found that the electrical conductivity was increased

1. *Ibid*, p. 99.

2. *Proc. Roy. Soc., B.* Vol. LXXVII, p. 267, 1906.

3. *Proc. Roy. Soc., B.* Vol. LXXVI, p. 322, 1906.

4. *Loc. cit.*, p. 278.

5. *Journ. Physiol.*, Vol. XXIII, proc. p. 11, 1899.

after the skin was subjected to the action of chloroform vapour.<sup>1</sup> He explains these results on a membrane hypothesis whereby the anaesthetic is supposed to act by altering a layer of lipoid surrounding each cell.<sup>2</sup> The presence of a resting current shows an unequal distribution of electrolytes in which the ions of the electrolytes are moving in such a direction that the difference of potential tends to be abolished. If the salts of the lymph are less firmly united to the proteins than are those of the epidermal cells, so that the active mass of electrolytes is greater at the inner than at the outer surface, then setting free of salts on the application of chloroform to the outer surface would diminish the electrical potential and at the same time increase the electrical conductivity. There is, therefore, nothing in Alcock's experiments which disproves that chloroform can detach electrolytes from combination or adsorption with proteins.

Stewart in working with muscle extracts found that heat coagulation caused an increase of electrical conductivity due to a detachment of electrolytes, but the effect on the molecular concentration was peculiar. Freezing point determinations showed sometimes a greater and sometimes a less depression after removal of the proteins. These changes were not due to experimental error, as the changes are beyond the error of the method used.<sup>3</sup>

Ringer<sup>4</sup> and Locke<sup>5</sup> showed the effect on the heart beat of various inorganic salts when perfused through the arteries, and suggested that a mixture of salts is more favourable than any individual constituent of the solution. Later observers have demonstrated that this rule holds true for every kind of cell activity. The results of experiments on the influence of various salts on cell activity have led to the ion-protein

1. *Proc. Roy. Soc., B.* Vol. LXXVIII, p. 159, 1906, in a foot note, p. 169, he states, 'Using the apparatus mentioned in the text, it was found that any alteration in the resistance of serum was not detectable after  $\text{CHCl}_3$ , but that there was a diminution of approximately 15 per cent. in the case of blood (laked or simply defibrinated); it would seem that the effect of  $\text{CHCl}_3$  on haemoglobin would be worth investigating.' Cf. Edie, *Thompson Yates and Johnston Laboratories Reports*, Vol. VI, Part I, p. 195, 1905.

2. E. Overton, *Studien über die Narkose*, Jena, G. Fischu, 1901, and Hans Meyer, *Arch. f. exper. Path. u. Pharm.*, Vol. XLII, p. 109, 1899, and Vol. XLVI, p. 338, 1901.

3. *Journ. Physiol.*, Vol. XXIV, p. 460, 1899.

4. Schäfers, *Text Book of Physiology*, Vol. II, p. 225, 1900.

5. *Journ. Physiol.*, Vol. XVIII, p. 332, 1895.

hypothesis put forward independently by J. Loeb<sup>1</sup> and Pauli<sup>2</sup> and elaborated by many other workers.

The present research was undertaken to see if anaesthetics and other toxic substances might so alter the chemical activities of proteins that electrolytes would be detached from the cells.

#### METHOD

The method employed was that of dialysis carried out in a similar manner to that used by Spence and Edie in determining the percentage of sugar in blood serum.<sup>3</sup>

It was not considered necessary to keep the cells intact, so instead of saline the dialysing fluid used was distilled water. This latter had several advantages. First, it broke up the cells by dialysing out the salts, thus minimising the effect of any impermeable membrane surrounding the cells. Secondly, it did not mask small differences in the salts dialysing out.

As putrefaction, by altering the proteins, would probably change the relationship of the proteins to the electrolytes, precautions against bacterial change were taken. The material was collected as freshly as possible from the animal, and all vessels or instruments with which it came in contact were sterilized. During the period of dialysis the experiments were conducted in an ice chest to reduce bacterial growth to a minimum.

The experiments were carried out as follows :—

A series of sterilized sausage dialysing tubes were placed in sterile wide-mouthed bottles. Equal portions (50 grammes or 50 c.c.) of the material under examination were put into these tubes and the tubes surrounded with equal bulks (100 c.c.) distilled water.

The first of these tubes was not treated further but was kept as a control. The other tubes were acted on in various ways. A known

1. J. Loeb, *Dynamics of Living Matter*, pp. 78-105, New York, 1906.

2. W. Pauli, *Ueber physikalisch Chemische Methode und Probleme in der Medizin*, Wien, 1900. *Cit. nach* Loeb.

3. *Bio-Chem. Jour.*, Vol. II, p. 103, 1907.

volume of chloroform or ether was added to certain of the bottles. In most cases an excess of these reagents was added so that the liquid would be completely saturated and the maximum effect obtained. Certain of the chloroform experiments were performed with smaller quantities to test the effect around the anaesthetising value. As the S.G. of  $\text{CHCl}_3 = 1.5$  and the total bulk of liquid =  $50 + 100 = 150$ , the number of cubic centimetres of chloroform added corresponds to the percentage of chloroform by weight in the liquid. The effect of coagulation of the proteins was studied by placing the bottles (containing the water, sausage tubes and material) into a water bath, heating to boiling and allowing the whole to remain at boiling temperature for ten minutes. Carbon dioxide was used by passing it from a Kipp through a wash bottle and allowing it to bubble through the dialysing fluid for about an hour. Finally in some cases the dialysing fluid instead of distilled water consisted of  $\frac{n}{50}$  acetic acid in distilled water. After the tubes had been treated the stoppers were inserted and the bottles placed in the ice chest.

After dialysis had proceeded for two days the bottles were taken from the ice chest, the sausage tubes were removed, and the volume of the dialysate was measured. The dialysate was evaporated to dryness and incinerated at a dull red heat. The ash from the dialysate was weighed, after cooling in a desiccator, and then it was dissolved in distilled water and made up to 100 c.c. Aliquot portions of this solution were taken for analysis. Ten cubic centimetres were titrated for chlorides with  $\frac{n}{10} \text{AgNO}_3$ , using potassium chromate as indicator. Twenty-five cubic centimetres were titrated with standard uranium acetate, using potassium ferrocyanide as an outside indicator. The 'reactivity' was determined in 10 c.c. by titrating with  $\frac{n}{10} \text{H}_2\text{SO}_4$  in the presence of phenol-phthalein. After the pink colour had disappeared di-methyl-amido-azo-benzol was added and the titration continued until the yellow colour changed to orange. The 'reactivity' to di-methyl. is the total amount of acid added from the beginning of



the titration. Finally the electrical conductivity was measured by the method of Kohlrausch, and in certain cases the presence of calcium, magnesium and sulphates was sought, but in none of the experiments were these present in sufficient amount to obtain quantitative measurements. The results obtained were corrected so that the figures given correspond to the amount of substance present in 100 c.c. of the original dialysate. Thus suppose that the original volume of the dialysate was 98 c.c. then, because the ash was made up to 100 c.c., the figures for 100 c.c. of the solution after incineration were multiplied by  $\frac{100}{98}$  to express the concentration in 100 c.c. of the original dialysate.<sup>1</sup>

The experiments are given under the following heads :—

- A. Experiments with Red Blood Corpuscles.
- B. Experiments with Serum.
- C. Experiments with Muscle.
- D. Experiments with Liver.
- E. Experiments with Kidney.
- F. Experiments with Brain.

#### A. EXPERIMENTS WITH RED BLOOD CORPUSCLES

For these experiments the blood was collected, at the slaughter-house, direct from an artery into a sterilized pail, and defibrinated by whipping with a sterile whisk. The blood was centrifugalised in sterile glasses and the corpuscles separated as completely as possible from the serum. All the corpuscles were thoroughly mixed, and 50 c.c. was placed into each of the sausage tubes and treated as mentioned in the tables. In some of the experiments an excess of chloroform and ether was used, but in addition in Experiments II and III smaller quantities of chloroform were added to see if the same results could be obtained around the anaesthetising value.

1. The correction in the case of ether is probably too high, as the solubility of ether in water is about 8 per cent. the amount of water in the dialysate would be  $\frac{102}{100}$  of the volume of the dialysate.

TABLE I. PIG'S RED BLOOD CORPUSCLES

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}H_2SO_4$ to Phenolphthalein	$\frac{N}{10}H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ... ..	88 c.c.	0.1320	0.0174	0.0403	1.7 c.c.	6.8 c.c.	0.002061
2. Chloroform—3 c.c. put into dialysing tube ... ..	85 c.c.	0.2532	0.0267	0.0710	3.5 c.c.	14.7 c.c.	0.004082
3. Ether—15 c.c. put into the dialysing tube ... ..	82 c.c.	0.2234	0.0320	0.0563	2.4 c.c.	12.2 c.c.	0.003378
4. Boiled ... ..	87 c.c.	0.1906	0.0302	0.0488	2.3 c.c.	10.3 c.c.	0.003023

TABLE II. PIG'S RED BLOOD CORPUSCLES

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}H_2SO_4$ to Phenolphthalein	$\frac{N}{10}H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ... ..	94 c.c.	0.0600	0.0093	0.0226	6.4 c.c.	1.91 c.c.	0.001216
2. Chloroform — 0.1 per cent. 0.1 c.c. of $CHCl_3$ put into the dialysing tube	88 c.c.	0.1329	0.0134	0.0383	1.38 c.c.	3.4 c.c.	0.002058
0.5 per cent. 0.5 c.c. $CHCl_3$ put into the tissue in the dialysing tube ...	88 c.c.	0.1100	0.0135	0.0323	3.4 c.c.	9.09 c.c.	0.001870
1 per cent. 1.0 c.c. $CHCl_3$ put into dialysing tube ...	89 c.c.	0.1178	0.0118	0.0379	3.15 c.c.	4.5 c.c.	0.002102
10 c.c. $CHCl_3$ put into dialysing tube	92 c.c.	0.1314	0.0138	0.0516	1.2 c.c.	3.26 c.c.	0.002372
3. Ether—10 c.c. inside and 20 c.c. outside the dialysing tube ...	102 c.c.	0.0404?	0.0086	0.0313	0.98 c.c.	1.96 c.c.	0.002139
4. Boiled ... ..	95 c.c.	0.1648	0.0166	0.0568	1.9 c.c.	4.42 c.c.	0.003200
5. $CO_2$ passed through the dialysing fluid	96 c.c.	0.0918	0.0074	0.0296	2.19 c.c.	5.83 c.c.	0.001656
6. $\frac{M}{50}$ Acetic acid ...	96 c.c.	0.1292	0.0105	0.0370	2.19 c.c.	5.20 c.c.	0.002160

TABLE III. PIG'S RED BLOOD CORPUSCLES

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}$ $H_2SO_4$ to Phenolphthalein	$\frac{N}{10}$ $H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ...	81 c.c.	0.1393	0.0161	0.0307	1.6 c.c.	6.17 c.c.	0.00222
2. Chloroform — 0.05 per cent. 0.05 c.c. of $CHCl_3$ put into dialysing tube ...	86 c.c.	0.1326	0.0167	0.0330	2.9 c.c.	5.81 c.c.	0.002242
0.1 per cent. 0.1 c.c. put into dialysing tube ...	85 c.c.	0.1480	0.0188	0.0334	2.49 c.c.	5.3 c.c.	0.002284
0.5 per cent. 0.5 c.c. put into dialysing tube ...	76 c.c.	0.1745	0.0268	0.0420	3.3 c.c.	7.2 c.c.	0.002967
1 per cent. 1.0 c.c. put into dialysing tube ...	88 c.c.	0.1168	0.0154	0.0343	2.35 c.c.	5.57 c.c.	0.0020207

In these tables it is shown that chloroform, ether, carbon dioxide, acetic acid and heat coagulation all influence the condition of the electrolytes in the corpuscles so that a larger amount dialyses out than when the dialysing fluid consists only of distilled water. That the results were not due to an action on a lipid membrane was shown by the fact that the controls were completely laked by dialysis. Under the microscope the 'ghosts' were seen embedded in a red semi-solid mass. No. 3, Table II, is apparently an exception, and the results with lower percentages in Table III are not so striking as those in Table II; but in Experiment III the corpuscles were not so fresh as usual, thus comparing with the 'spontaneous' laking of Stewart.<sup>1</sup>

1. *Journ. Physiol.*, Vol. XXIV, p. 230, 1899.

## B. EXPERIMENTS WITH SERUM

The experiments under this section were performed under similar conditions to those in Section A except that, instead of using 50 c.c. of corpuscles, 50 c.c. of serum was in each case put into the dialysing tubes.

TABLE IV. PIG'S SERUM

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}H_2SO_4$ to Phenolphthalein	$\frac{N}{10}H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ...	95 c.c.	0.2623	0.0079	0.1270	4.2 c.c.	6.8 c.c.	0.004853
2. Chloroform — 0.1 c.c. put into dialysing tube ...	94 c.c.	0.2254	0.0065	0.1170	3.19 c.c.	4.8 c.c.	0.04413
0.5 c.c. put into dialysing tube ...	95 c.c.	0.2284	0.0055	0.1121	3.2 c.c.	5.3 c.c.	0.004534
1 c.c. put into dialysing tube ...	96 c.c.	0.2416	0.0055	0.1146	2.08 c.c.	3.3 c.c.	0.00437
10 c.c. inside the dialysing tube ...	94 c.c.	0.2594	0.0075	0.1246	3.19 c.c.	5.3 c.c.	0.004574
3. Ether—10 c.c. inside, 20 c.c. outside, dialysing tube ...	108 c.c.	0.2204 (0.24 <sup>1</sup> )	0.0065	0.1085	2.7 c.c.	4.6 c.c.	0.004175 (0.0045 <sup>1</sup> )
4. Boiled ...	96 c.c.	0.2477	0.0073	0.1183	3.1 c.c.	5.2 c.c.	0.004477
5. <sup>2</sup> CO <sub>2</sub> as before ...	97 c.c.	0.2318	—	—	—	—	—
6. $\frac{M}{50}$ Acetic acid ...	95 c.c.	0.2823	0.0092	0.1233	5.3 c.c.	10.5 c.c.	0.005017

TABLE V. PIG'S SERUM

Result of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}H_2SO_4$ to Phenolphthalein	$\frac{N}{10}H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ...	95 c.c.	0.2562	0.0084	0.1196	3.79 c.c.	6.31 c.c.	0.004937
2. Chloroform—10 c.c. inside dialysing tube ...	93 c.c.	0.2546	0.0096	0.1223	4.42 c.c.	6.6 c.c.	0.004949
3. Ether—10 c.c. inside, 20 c.c. outside, dialysing tube ...	103 c.c.	0.2704	0.0077	0.0966	2.91 c.c.	4.85 c.c.	0.004154 (0.0045 <sup>3</sup> )
4. Boiled ...	96 c.c.	0.2852	0.0083	0.1246	4.17 c.c.	6.25 c.c.	0.004929
5. CO <sub>2</sub> passed through for an hour ...	95 c.c.	0.2686	0.0096	0.1196	5.26 c.c.	7.58 c.c.	0.005020
6. $\frac{M}{50}$ Acetic acid ...	95 c.c.	0.2867 <sup>4</sup>	0.0084	0.1196	4.00 c.c.	6.00 c.c.	0.004795

1. See footnote, p. 417.

2. In No. 5 the porcelain dish broke during incineration, so that only the total salts could be estimated.

3. See footnote, p. 417.

4. All the carbon could not be properly burnt off in this experiment owing to the vessel cracking during the incineration.



The results in these cases were negative, thus contrasting with the experiments described by Moore and Roaf. In their experiments, however, the chloroform was added to the undiluted serum, whilst in the present experiments the dialysis would correspond to a two-thirds dilution with water. If the salts are so loosely united to the proteins that they are detached by mere dilution the negative results obtained by us would be easily explained. That the salts are detached from proteins by dilution has been shown by Stewart,<sup>1</sup> and more recently by Mellanby.<sup>2</sup>

### C. EXPERIMENTS ON MUSCLE

The muscle used for these experiments was dissected as free as possible from fat, and it was then ground up in a mincing machine. Fifty grammes of this material was placed into each of the sausage tubes and they were then treated as in the preceding experiments. The rabbits' muscle was obtained from a couple of freshly-killed rabbits, but that of the ox was obtained from a butcher's shop, and hence was neither so fresh nor so sterile as the former.

TABLE VI. RABBITS' MUSCLE

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}$ $H_2SO_4$ to Phenolphthalein	$\frac{N}{10}$ $H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ... ..	91 c.c.	0.2786	0.0243	0.0195	3.63 c.c.	14.29 c.c.	0.003143
2. $CHCl_3$ —10 c.c. put into the dialysing tube ... ..	89 c.c.	0.3239	0.0259	0.0248	5.62 c.c.	18.66 c.c.	0.00395
3. Ether—20 c.c. put inside dialysing tube, 10 c.c. into the dialysing water	91 c.c.	0.3356	0.0296	0.0195	5.82 c.c.	19.67 c.c.	0.00372
4. Boiled 10 mins. ...	89 c.c.	0.3868	0.0314	0.0248	7.76 c.c.	22.96 c.c.	0.0044

1. *Journ. Physiol.*, Vol. XXIV, p. 217, 1899.

2. *Journ. Physiol.*, Vol. XXXV, pp. 193-196, 1907.

TABLE VII OX MUSCLE

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}$ $H_2SO_4$ to Phenolphthalein	$\frac{N}{10}$ $H_2SO_4$ to Dimethyl	Specific conductivity $K \times 10^2$
1. Control ...	88 c.c.	0.2207	0.0477	0.0141	5.68 c.c.	15.9 c.c.	0.003392
2. Chloroform—10 c.c. inside the dialysing tube ...	92 c.c.	0.2207	0.0469	0.0161	6.5 c.c.	15.2 c.c.	0.003205
3. Ether—10 c.c. inside 20 c.c. outside the dialysing tube ...	102 c.c.	0.3218	0.0523	0.0167	5.9 c.c.	15.7 c.c.	0.00342
4. $\frac{M}{50}$ Acetic acid ...	91 c.c.	0.2956	0.0529	0.0156	9.9 c.c.	21.4 c.c.	0.00373

These both show an increase above the control in all except No. 2, Table VII.

#### D. EXPERIMENTS ON LIVER

These experiments were performed similarly to those with muscle. Fifty grammes of the material minced and dissected free from visible fat was placed into each tube.

TABLE VIII. LIVER

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}$ $H_2SO_4$ to Phenolphthalein	$\frac{N}{10}$ $H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ...	94 c.c.	0.1496	0.0307	0.0226	1.91 c.c.	6.7 c.c.	0.001656
3. Ether—20 c.c. inside, 10 c.c. outside, the dialysing tube ...	103 c.c.	0.1800	0.0383	0.0241	3.69 c.c.	9.5 c.c.	0.001858
4. Boiled ...	93 c.c.	0.1938	0.0452	0.0263	3.23 c.c.	8.6 c.c.	0.002609
6. $\frac{M}{50}$ Acetic acid ...	96 c.c.	0.2048	0.0456	0.0188	3.1 c.c.	10.63 c.c.	0.002438

TABLE IX. LIVER

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}H_2SO_4$ to Phenolphthalein	$\frac{N}{10}H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ...	94 c.c.	0.1432	0.0340	0.0226	1.4 c.c.	6.4 c.c.	0.001804
2. Chloroform—5 c.c.	92 c.c.	0.1276	0.0326	0.0193	2.2 c.c.	6.85 c.c.	0.001900
1 c.c. ...	94 c.c.	0.1250	0.0276	0.0200	2.02 c.c.	5.64 c.c.	0.00175
10 c.c. inside dialysing tube ...	92 c.c.	0.1365	0.0409	0.0240	2.2 c.c.	6.2 c.c.	0.002017
3. Ether—10 c.c. inside, 10 c.c. outside, dialysing tube	103 c.c.	0.1324 (0.144 <sup>1</sup> )	0.0390	0.0197	1.94 c.c.	5.83 c.c.	0.0017000 0.00185 <sup>1</sup>
4. Boiled ...	93 c.c.	0.0553 (0.1553 <sup>2</sup> )	0.0383	0.0267	2.15 c.c.	6.8 c.c.	0.002209

Another experiment on liver tissue shows an increase of electrolytes in the treated tubes, but, as all the analyses, etc., were not complete, the table is not shown.

Table VIII shows that the treatment to which the liver tissue was subjected caused a liberation of salts, but Table IX shows no such increase. A further experiment in which the analyses were not all complete, and hence not worth publishing, shows a detachment of salts by the treatment adopted.

#### E. EXPERIMENTS WITH KIDNEY

The kidney for this experiment was obtained from a pig, and cleared from visible fat in the usual way. After mincing, fifty grammes of the tissue was placed into each sausage tube and treated in the usual manner.

1. See footnote, p. 417.

TABLE X. KIDNEY

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate.

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}$ $H_2SO_4$ to Phenolphthalein	$\frac{N}{10}$ $H_2SO_4$ to Dimethyl	Specific conductivity
1. Control, as before	94 c.c.	0.1117	0.0121	0.0378	1.06 c.c.	4.26 c.c.	0.00224
2. Chloroform — 10 c.c. inside ...	95 c.c.	0.1406	0.0243	0.0374	2.10 c.c.	6.32 c.c.	0.002571
3. Ether—10 c.c. inside, and 10 c.c. 15 c.c. outside ...	105 c.c.	0.17105	0.0150	0.0345	1.90 c.c.	4.76 c.c.	0.001993 (0.00217) <sup>1</sup>
4. Boiled ...	93 c.c.	0.2447	0.0322	0.0791	3.25 c.c.	9.14 c.c.	0.004103
5. $CO_2$ ...	95 c.c.	0.1307	0.0166	0.0345	1.58 c.c.	4.74 c.c.	0.002568
6. $\frac{M}{50}$ Acetic acid, as before ...	96 c.c.	0.1242	0.0201	0.0370	1.98 c.c.	4.38 c.c.	0.002174

## REMARKS

In this experiment the percentage of chlorine was found to be lower than the control in all except the boiled one, but this slight apparent lowering is due to the differences in volume of the dialysates, so lowering the percentages. The actual result of the analysis of the chlorine on taking 10 c.c. of each fluid was—1 c.c. of silver nitrate in each case except in the boiled.

In this experiment the chlorides showed no increase over the control, but all the methods of treatment showed an increase of total salts, phosphates and reactivity, and all save the ether and acetic acid tubes showed an increase of electrical conductivity.

## F. EXPERIMENTS WITH SHEEP'S BRAINS

The heads of the sheep were brought up from the slaughter-house and the brains (cerebrum, cerebellum and medulla) dissected out. The larger blood vessels were removed and the whole of the brain matter minced. Fifty grammes of the minced material was put into each sausage tube and treated as usual. In Table XI the actual results of the analysis are given, and in Table XII the same experiment is shown corrected as in the other tables.

1. See footnote p. 417.



TABLE XI. SHEEP'S BRAIN

This table shows the actual figures of the quantities taken for titration, etc., obtained in the analysis of the incinerated dialysate after being made up to 100 c.c. with distilled water, before being corrected, so as to represent percentages of the original dialysate.

Result of the analysis of the incinerated dialysate made up to 100 c.c. with distilled water representing the actual figures for quantities taken.

Method of Treatment	Volume of dialysate	Total salts in grams	10 c.c. of dialysate taken to titrate for Chlorides AgNO <sub>3</sub>	20 c.c. of dialysate taken to titrate for the Phosphates <sup>1</sup> Uran. Acet.	10 c.c. dialysate taken to titrate with $\frac{N}{10}$ H <sub>2</sub> SO <sub>4</sub> to Phenol-phthalein $\frac{N}{10}$ H <sub>2</sub> SO <sub>4</sub>	10 c.c. dialysate taken to titrate $\frac{N}{10}$ H <sub>2</sub> SO <sub>4</sub> to dimethyl-amido-oxo-benzole $\frac{N}{10}$ H <sub>2</sub> SO <sub>4</sub>	The resistance in ohms of the cell of a resistance capacity = 0.67196
1. Control ... ..	95 c.c.	0.0616	0.66 c.c.	0.4 c.c.	0.2 c.c.	0.35 c.c.	770 ohms
2. Chloroform — 10 c.c. put into dialysing tube ...	95 c.c.	0.1154	1.8 c.c.	0.72 c.c.	0.23 c.c.	0.70 c.c.	372 „
3. Ether—20 c.c. inside, 10 c.c. outside	109 c.c.	0.1234	2.20 c.c.	0.87 c.c.	0.38 c.c.	0.74 c.c.	358 „
4. Boiled ... ..	97 c.c.	0.2194	3.32 c.c.	1.60 c.c.	0.46 c.c.	1.08 c.c.	218 „
5. CO <sub>2</sub> ... ..	95 c.c.	0.1298	1.98 c.c.	0.64 c.c.	0.3 c.c.	0.52 c.c.	357 „
6. $\frac{M}{50}$ Acetic acid ...	96 c.c.	0.1238	2.00 c.c.	0.65 c.c.	0.2 c.c.	0.6 c.c.	394 „

TABLE XII. SHEEP'S BRAIN

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as P <sub>2</sub> O <sub>5</sub> in grams	Chlorine in grams	$\frac{N}{10}$ H <sub>2</sub> SO <sub>4</sub> to Phenol-phthalein	$\frac{N}{10}$ H <sub>2</sub> SO <sub>4</sub> to Dimethyl	Specific conductivity
1. Control ... ..	95 c.c.	0.0648	0.0092	0.0123	2.10 c.c.	3.58 c.c.	0.000919
2. Chloroform—10 c.c. inside the dialysing tube ...	95 c.c.	0.1215	0.0166	0.0134	2.42 c.c.	7.37 c.c.	0.001902
3. Ether—20 c.c. inside the dialysing tube, but 10 c.c. outside ... ..	109 c.c.	0.1132	0.0175	0.0358	3.49 c.c.	6.79 c.c.	0.001603
4. Boiled ... ..	97 c.c.	0.2262	0.0361	0.0608	4.75 c.c.	11.13 c.c.	0.003179
5. CO <sub>2</sub> passed through the 100 c.c. of distilled water for 1 hour ... ..	95 c.c.	0.1289	0.0136	0.0370	2.09 c.c.	5.42 c.c.	0.001766
6. $\frac{M}{50}$ Acetic acid ...	96 c.c.	0.1366	0.0150	0.0368	3.16 c.c.	6.32 c.c.	0.001981

1. Notice that 20 c.c. of dialysate was taken here instead of 25 c.c., which was taken in the other experiments. This method of illustrating the results shows well the marked difference between the control and the other bottles. Notice the large volume of the ether dialysate, the results of which have to be corrected to represent them in percentages, thus materially lowering the figures (see footnote, p. 417).

TABLE XIII. SHEEP'S BRAIN

Results of analysis of the dialysate after being incinerated and made up to 100 c.c. again, corrected so as to represent actual figures for 100 c.c. of original dialysate

Method of Treatment	Volume of dialysate	Total salts in grams	Phosphorus as $P_2O_5$ in grams	Chlorine in grams	$\frac{N}{10}H_2SO_4$ to Phenolphthalein	$\frac{N}{10}H_2SO_4$ to Dimethyl	Specific conductivity
1. Control ...	94 c.c.	0.1743	0.0343	0.0216	2.74 c.c.	9.47 c.c.	0.003463
2. Chloroform — 0.5 per cent. 0.5 c.c. put inside dialysing tube ...	95 c.c.	0.1690	0.0336	0.0463	4.42 c.c.	8.63 c.c.	0.003144
1 per cent. 1 c.c. put inside dialysing tube ...	96 c.c.	0.1800	0.0354	0.0481	4.17 c.c.	8.86 c.c.	0.003226
2 per cent. 2 c.c. put inside dialysing tube ...	93 c.c.	0.2090	0.0505	0.0657	6.45 c.c.	14.00 c.c.	0.00427
3. Ether—10 c.c. inside, 20 c.c. outside, dialysing tube	104 c.c.	0.2167	0.0679	0.0679	3.85 c.c.	10.58 c.c.	0.004038

In these experiments also there is an increase of inorganic salts in the dialysate after treatment. In the experiment with smaller quantities of chloroform it must be remembered that the large quantity of lipid material would dissolve the anaesthetic so that the vapour pressure would be lowered in the mixture, thus corresponding to a smaller active mass of chloroform acting on the protein.

#### DISCUSSION OF RESULTS AND THEORETICAL CONSIDERATIONS

The tables show that, in the great majority of the experiments, agents which diminish the activity of the cells cause a change in the physical condition of the contained electrolytes. This change consists in a setting free of the ions so that they are able to dialyse through parchment paper in greater numbers than in an untreated control.

The great interest of these results lies in the explanation they afford of the normal distribution of the inorganic constituents of the body to the different tissues and in the action of certain toxic substances on this distribution. The whole body is supplied by a common

nutrient medium, the blood plasma, and from this circulating medium the tissue cells pick out the particular constituents needful for their proper functioning. As the inorganic constituents of cells differ markedly in their percentage composition from the corresponding salts in the serum, there must be some mechanism which prevents the diffusion of salts from a position of higher concentration to that of lower concentration.

That this barrier to diffusion consists of an impermeable membrane is unlikely, as such a barrier would prevent fresh material being taken up for growth, and thus one would need to suppose that the ovum contains sufficient salts for the fully developed adult. A membrane possessing one-sided permeability is not known outside of biological processes, and thus the difference of composition between cells and their surrounding medium cannot, at present, be explained as a purely physical process. It is further probable that the salts are not held in the cell by a membrane, because in the experiments on red blood corpuscles the laking was complete, as was shown by examining the corpuscular mass under the microscope. The difference between the control and the treated specimens could not then be due to the destruction of an impermeable membrane<sup>1</sup>.

It is either necessary to suppose that the cells transform energy from chemical processes into osmotic manifestations of energy, and thus they keep the concentration of certain salts higher inside their boundaries than in the lymph surrounding them; or that the proteins or other constituents of the cells adsorb or combine with particular ions. An equilibrium is thus maintained between the free ions in the cells and in the plasma, although the percentage of some ions is much higher inside the cells.

On such a view, that the selection of particular salts by cells is regulated by the affinity of some constituent for the desired ions, one would expect to find that the salts of the nutrient medium would be quite free or only loosely combined. Our experiments show that

1. Stewart, *Journ. Physiol.*, Vol. XXIV, p. 230, 1899: he says that the salts are retained in the ghosts after blood corpuscles are laked but this does not necessarily mean that they are retained by a membrane. A membrane which permits the escape of haemoglobin would very likely be permeable to inorganic salts.

for serum this is the case, as dialysis into a comparatively small bulk of liquid separates nearly all the salts. It is difficult to say whether the detachment of electrolytes has anything to do with the causation of anaesthesia, but in view of the ion-protein hypothesis it is a very tempting deduction. The agents used in the present experiments were those that are known to cause narcosis. Chloroform, ether, and carbon dioxide all cause anaesthesia, whilst in diabetes, coma is due to an increased formation of organic acids which may act in a comparable manner to the acetic acid used in these experiments. In most cases the amount of narcotic used was greatly in excess of that necessary to cause anaesthesia, but with lesser amounts the action would probably be of the same nature only lesser in degree.

Heat coagulation is a more marked form of injury, and one from which recovery is not possible, as in the other modes of treatment employed, but it would be interesting if some of the symptoms of hyperpyrexia were due to a similar detachment of electrolytes.<sup>1</sup>

The diminished activity due to anaesthetics, if caused by a redistribution of electrolytes, can easily be recovered from, as removal of the active agent would allow the normal relations to be re-established. This explanation would also hold for unicellular and multicellular plants and animals. To test this hypothesis on living tissues is extremely difficult. The salts of any perfusion fluid would mask small differences due to the narcotic, and even if salts are liberated from the protein<sup>2</sup> it is not likely that they would immediately pass into the blood vessels. The change in the condition of the salts would occur inside the cell, and the ions would have to diffuse into the surrounding lymph before they could pass into the blood vessels. For these reasons there is not much hope of being able to find any output of salt into a perfusion fluid as the result of the action of an anaesthetic on surviving tissues.

Considering the experimental conditions, it is remarkable that

1. Cf. Bayliss, *Bio-Chem. Journ.*, Vol. I, pp. 191 and 192, 1906, and J. Mellanby, *Journ. Physiol.*, Vol. XXXV, p. 492, 1907.

2. See Vernon, *Zeit. f. allg. Physiol.*, Vol. VI, p. 393, 1907, in which he shows that chloroform and ether, when perfused through the blood vessels of kidneys, cause an increased detachment of protein.



the results show such an **increase** of salts in the treated tubes above the amount diffusing out from the controls. The controls probably have a fair amount of salt detached by the dialysis lowering the concentration of the electrolytes in equilibrium with the combined salts, and any putrefactive change, to which the controls are more liable owing to the absence of preservatives such as chloroform and ether, would tend to liberate salts.<sup>1</sup>

It is not intended to review the voluminous literature dealing with the relation of various salts to protein solutions, but there are one or two observations which are of value in connection with the present experiments.

The work of Macdonald has shown that injury and death of nerves liberates electrolytes.<sup>2</sup> He has also shown that nerves which are placed in saline containing chloroform tend to have the potassium salts limited in their distribution.<sup>3</sup> Bayliss, in working at the adsorption of dyes by cellulose, found that electrolytes greatly affected the amount adsorbed,<sup>4</sup> and Robertson has brought forward evidence that infusoria stain differently according to the nature of the salt in which they have been grown.<sup>5</sup> Mayr showed that certain salts affect the staining of the Nissl granules by toluidin blue,<sup>6</sup> and Hamilton Wright found that administration of chloroform and ether caused the Nissl granules to lose their affinity for methylene blue.<sup>7</sup>

From the evidence of these observers it would appear as if during anaesthesia there is some change in the condition of the electrolytes inside the cells, and it is not improbable that it is this change carried to an extreme degree which accounts for the results given in this paper.

1. Stewart, *Journ. Physiol.*, Vol. XXIV, p. 230, 1899.

2. *Loc. cit.*

3. *Journ. Physiol.*, Vol. XXXV., proc. p. 38, 1907.

4. *Bio-Chem. Journ.*, Vol. I, p. 175, 1906.

5. *Journ. Biolog. Chem.*, Vol. I, p. 279, 1906.

6. *Beit. z. Chem. Physiol. u. Path.*, Vol. VII, p. 548, 1906. See also Bethe, *ibid.*, Vol. VI, p. 399, 1905.

7. *Journ. Physiol.*, Vol. XXVI, pp. 30 and 362, 1901-1902.

## SUMMARY

1. Chloroform, ether, carbon dioxide, acetic acid and heat coagulation cause a change in the physical condition of the inorganic constituents of tissues.

2. This change consists in a liberation of ions so that they are able to diffuse through parchment in greater number than before treatment with these reagents.

3. The tissues which detach salts under these conditions are red blood corpuscles, brain, liver, muscle and kidney, but blood serum does not show any appreciable difference when acted upon by the various reagents.

4. It is believed that the setting free of electrolytes is not due to the reagents acting on an impermeable membrane surrounding the cells, but is the result of an alteration in the relationship of the salts to the proteins.

5. The bearing of these changes on the activity of cells is briefly discussed.

We wish to thank Prof. Benjamin Moore for valuable advice during the course of our experiments.

## ON SOME APPLICATIONS OF SAFRANIN AS A TEST FOR CARBOHYDRATES

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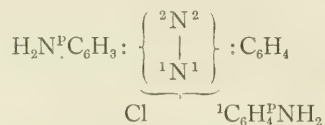
Safranin was first suggested as a test for sugar by Crismer<sup>1</sup>, who  
 1. *Pharm. Zeit.*, Vol. XXXIII, p. 65; *Pharm. Journal* [3], Vol. XIX, p. 348.  
 recommended it as a suitable reagent for the detection of glucose in urine. Since that time the reagent has been employed by various observers, but no satisfactory conclusion with regard to the exact usefulness of the test from the clinical standpoint has been forthcoming. For certain purposes, however, as will be shown below, it seems to hold a very definite position, while, on the other hand, its general usefulness can only be appreciated after certain disadvantages are understood and allowed for.

Safranin occurs in commerce as a reddish or brownish-red powder, readily soluble in water to form a blood-red solution which does not readily undergo decomposition on keeping. The addition of caustic alkali causes no immediate precipitate if the alkali is not too concentrated, but there is a tendency for the formation of a precipitate on standing; the stronger the alkali, the more readily a precipitate separates out. If a solution of glucose be added to the alkaline solution and the mixture heated to the boiling point, the red colour is discharged, and the liquid changes to a pale straw-yellow colour; in the presence of a good deal of sugar it becomes turbid from the separation of the insoluble leuco-derivative. In the presence of small amounts of sugar the red colour returns on agitating the liquid or exposing it to the air.

Commercial safranin seems to vary a good deal in composition, but according to Allen<sup>1</sup> is a mixture of several homologous bodies, of

1. *Chemistry of Urine*, 1895, p. 83.

which  $C_{19}H_{17}N_4Cl$ ,  $C_{20}H_{19}N_4Cl$ , and  $C_{21}H_{21}N_4Cl$  are the chief. The second of these has the constitution of a chloride of paramido-phenyl-paramido-phenazonium, and is said to have the following structural formula :—



Unlike many other tests for sugar, safranin is absolutely unaffected by uric acid or urates, hippuric acid, creatinin, creatin, chloral, chloroform, hydrogen peroxide, salts of hydroxalamine, pyrocatechin or hydroquinone ; in short, the reaction seems to be entirely limited to substances of a carbohydrate nature. The following carbohydrate materials have been tested, the results being indicated by a plus sign where reduction was obtained, and by a minus sign where no reaction was given.

#### POLYSACCHARIDS

Starch — Ordinary samples may give a slight reaction but purification indicates that the reduction is due to impurities.

Dextrin + Sample used was obtained from Merck ; after precipitating several times with alcohol a well-marked reaction was still obtained.

Glycogen + Not very well marked reaction.

#### DISACCHARIDS

Cane sugar +

Maltose +

Lactose +

Iso-maltose + (?)

#### MONOSACCHARIDS

Dextrose +

Laevulose +

Galactose +

#### PENTOSES AND OTHERS

Xylose +

Arabinose +

Raffinose — (?) very slight reaction, probably due to impurities.

Glycuronic acid + gives very marked reaction.

Mucin —

Mucin boiled with HCl = glucosamine hydrochloride ; gives distinct reaction.



The above results indicate that safranin is essentially a reagent for indicating the presence of carbohydrate in general, and is of itself quite unsuitable for determining the presence of glucose in a mixture where other carbohydrates may be present. Every urine gives a fairly distinct reaction with safranin, and Crismer interpreted this as proving the presence of sugar in normal urine; if 'sugar' be used in the sense of carbohydrate matter in general, this seems to be true, but the nature of the carbohydrate is not directly indicated. The most marked use of the substance seems to be for the detection of small traces of carbohydrate matter, and for this purpose it seems to be the best and most easily applied reagent. The only important substance outside the carbohydrate group that interferes with the reaction is albumin. It is stated by Allen that albumin, while decolorising safranin but very slowly, yet, in the end, caused complete discharge of the red colour. This statement has been generally accepted and is still held by recent observers.

Albumin, however, has but little effect in causing a decolorisation of safranin, and the statement that long boiling causes complete discharge of the colour seems to be quite unfounded. If egg albumin is used, there is, of course, a certain amount of carbohydrate present as well, which no doubt is mainly responsible for any slight reduction apparently caused by the protein. Pure albumin appears to have no appreciable reducing action, and prolonged boiling with protein matter alone never results in the complete discharge of the red colour. When a fluid containing albumin is mixed with the alkaline safranin solution the mixture assumes a pinkish tint, as against the ordinary reddish colour, and this seems to be the most characteristic action of protein material on safranin. Solutions of alkaline safranin with albumin, after boiling for from thirty to sixty minutes, did not discharge the colour from even weak solutions of the reagent, and it would seem that the alleged direct reducing action of albumin, if it exists at all, is so insignificant as to render it of no importance, even in the case of comparatively weak safranin solutions.

For the detection of small amounts of sugar, however, albumin must be removed, for it tends to inhibit somewhat the reducing power

of the sugar on safranin ; a solution of glucose which completely discharges the colour from a certain amount of safranin will not give nearly so marked a reaction if some albumin be previously added to the mixture. In this case it will decolorise less safranin than in a protein-free solution, and for quantitative examination especially it is of importance to remove any protein that may be present in the solution before adding the safranin.

Another substance which somewhat interferes with the reaction is ammonia ; if the latter is present in excess it acts in much the same manner as albumin and prevents the sugar from discharging the red colour as completely as it would otherwise do.

#### THE REACTION OF NORMAL URINE WITH SAFRANIN

Every urine gives a more or less definite reaction with safranin as a result of the carbohydrate material present in normal urine. In order that the test should prove of practical utility for the detection of pathological amounts of sugar in urine, it is necessary to make allowance for this normal reaction ; and a lack of proper appreciation of the intensity of this reaction given by urine from healthy persons has undoubtedly helped to bring discredit on the test and to give rise to much confusion. By means of safranin very accurate determinations of the amount of carbohydrate material present in normal urine can be made with comparatively small amounts of urine. For a rough quantitative examination of the amount of reducing substances present in a sample of urine it is customary to use a 0.1 per cent. solution of safranin in water ; 1 c.c. of this solution is added to 1 c.c. of sodium hydroxide (5 per cent.) and the mixture is shaken up with 1 c.c. of the urine to be tested, and heated to boiling point ; if sugar is present in sufficient amount the red colour is discharged and the solution becomes yellow. In performing the test care should be taken to agitate the liquid as little as possible in order to prevent reoxidation of the reduced leuco-derivative. The general statement that 1 c.c. of a 0.1 per cent. solution of safranin is reduced by 1 c.c. of a 0.1 per cent. solution of chemically pure glucose is really inaccurate. In several samples of pure glucose obtained from Merck and from

Grübler it was found that from 1.5 c.c. to 2 c.c. of 0.1 per cent. solution of safranin was decolorised by 1 c.c. of a 0.1 per cent. solution of glucose ; different samples of safranin were used. In short, 1 c.c. of glucose solution will decolorise more nearly 2 c.c. of the same strength of safranin than 1 c.c., as generally stated. In the quantitative estimation of sugar it is quite impossible to obtain even an approximately quantitative indication of the amount present by adding safranin to the hot alkaline sugar solution as long as it is decolorised and subsequently calculating the amount of safranin solution used in terms of glucose. This simple plan has been advocated, but so many disturbing factors enter into the calculation as to render the results very inaccurate, and by no means constant for the same solution. Material differences are obtained as a result of the different periods of time taken to add the necessary amounts of safranin to the sugar solution ; for a considerable quantity of the sugar is destroyed by the hot caustic alkali and, naturally, the greater the interval between each addition of safranin the lower the total amount of sugar present appears to be. Again, the gradual addition of safranin causes agitation of the fluid, and this increases the tendency to reoxidation to such an extent as to very markedly interfere with the result. Further, only the roughest indication of the sugar actually present can be obtained by the above method. In short, safranin is not a satisfactory reagent for quantitative examination of fluids containing a large amount of sugar, since quicker and more accurate results can be obtained by other means ; on the other hand, it gives a rough general indication. For the estimation of such small amounts of sugar as cannot be satisfactorily ascertained by other tests, safranin, when carefully used, is, in the writer's opinion, one of the most useful of reagents, being both accurate and easy of application when special precautions are adopted ; its delicacy renders it of great value in ascertaining slight changes in the total amount of carbohydrate present in any fluid as the result of the reaction of a reagent or ferment.

By means of this test the presence of a distinct quantity of fermentable sugar (which may be taken as glucose) can be easily indicated in every urine, and this method affords an easy means of demonstrating



the presence of glucose in normal urine to a class of students. The quantity of fermentable sugar present in ordinary urine is so small as to render the results of fermentation exceedingly doubtful; any  $\text{CO}_2$  that is evolved is so slight in amount as to be easily held in solution by the fluid; again, if there is any evidence of fermentation having taken place, there is practically no clue obtained as to the amount of sugar actually present in terms of the  $\text{CO}_2$  evolved.

With safranin, however, the exact amount of fermentable sugar present can be easily determined, and the following simple plan for showing the presence of sugar in urine is quite striking in regard to the constancy of the results obtained.

About 50 c.c. urine is well mixed with some bakers' yeast and placed in the incubator at  $40^\circ \text{C}$ . for from eighteen to twenty-four hours. In order to keep the yeast thoroughly mixed with the solution it is shaken up at intervals. After fermentation has taken place the yeast is filtered off, and the necessary corrections made to allow for evaporation. A comparison of the fermented urine with a specimen of the same urine unfermented at once reveals the fact that the reducing power of the fermented specimen is now much less than that of the unfermented part. For example, a urine of which 1 c.c. completely decolorised about twenty-four drops of a 0.1 per cent. safranin solution before fermentation, was changed by fermentation to such an extent that under similar conditions 1 c.c. now decolorised only about eleven drops.

Besides reducing fermentable sugar, urine contains a certain proportion of unfermentable carbohydrate, for in no instance has it been possible to obtain a urine which gave no reaction with safranin after fermentation; in fact, the unfermentable residue is generally sufficient to give a fairly pronounced reaction. This seemed a good and easy method for determining the relative quantities of fermentable and non-fermentable carbohydrate-like substances in urine, but the question whether the apparently unfermentable substance might not be really fermentable sugar which had escaped fermentation owing to the inhibiting action of some of the normal constituents of urine had first to be answered. Again, it was possible that the products of



yeast ferment activity might render it impossible to eliminate the last traces of fermentable sugar in even a watery solution.

In order to test this, weak solutions of sugar (from 0.05 per cent. to 0.2 per cent.) were made up in distilled water and fermented with yeast in an open flask placed in the incubator at 40° C. Similar solutions containing as nearly as possible the correct percentage of the chief constituents of normal urine were made up and tested in the same way. In every case it was found that for practical purposes the sugar was entirely destroyed, or if there was any reaction it was so slight as to be of little importance.

The following results were obtained with above solutions, 1 c.c. of the fermented fluid being mixed with 1 c.c. sodium hydrate solution: the number of drops of safranin solution decolorised shows the extent to which fermentation had gone on.

Solution				No. of hours fermented	No. of drops of 0.7% safranin solution decolorised after fermentation
Glucose 0.05 % in dist. H <sub>2</sub> O	...	...	...	18	1.5
„ 1 % „	...	...	...	23	1
„ 1.5 % „	...	...	...	21	1.5
„ 0.05 % + urea	...	...	...	24	2
„ 1 % + „	...	...	...	22	2
„ 1.2 % + uric acid (alkaline fluid)	...			19	2
				18	2
„ 1.2 % + chlorides	...	...	...	18	2.5
„ 1.2 % + phosphates	...	...	...	22	2
„ 1.2 % + sulphates	...	...	...	21	2.5
„ 1.5 % + mixture of ordinary urine constituents	...			20	2

From the above it is obvious that none of the ordinary constituents of urine have any appreciable effect in inhibiting the action of yeast or in preventing its activity in the presence of very small amounts of sugar.

The effect of small amounts of acid and alkali was also investigated. The results obtained seemed to indicate that fermentation was accelerated to some slight extent by the presence of a very faint trace

of alkali in the sugar solution. On the other hand, small amounts of acid or alkali, sufficient to give a very distinct litmus reaction to the fluid, did not inhibit the reaction to any appreciable extent. With strong acids and alkalies my results were in agreement with those obtained by Drabble and Scott<sup>1</sup>—fermentation was either retarded or completely prevented. In testing the above safranin was exceedingly useful, for it is obvious that in alkaline fluids no importance can be attached to the absence of CO<sub>2</sub>, the gas, if evolved, being rapidly absorbed by the alkaline medium.

Again, some experiments were performed in which the urine was evaporated to a half, a third, and a quarter its bulk. If the residual substance was really an unfermentable carbohydrate the residue after fermentation should bear a constant and inverse ratio to the artificial concentration of the urine—a urine evaporated to say one-third of its bulk should, after fermentation, show three times as much residual substances as the same urine before concentration. This was always found to be the case, so that the substance remaining after fermentation seems really to be an unfermentable carbohydrate body.

ON THE TOTAL AMOUNT OF CARBOHYDRATE IN NORMAL  
URINE AND THE RELATIONSHIP OF THE UNFERMENTABLE  
TO THE FERMENTABLE PART

Certain experiments were performed with safranin in order to determine accurately the total amount of carbohydrate present in normal urine, calculated as glucose. A series of solutions of chemically pure glucose were made up in strengths of '02 per cent., '03 per cent., etc., up to '2 per cent., 1 c.c. of each of these solutions was mixed with 1 c.c. 5 per cent. NaOH and to this was added such an amount of safranin as was readily decolorised by the amount of sugar present after the mixture was heated for some time. In order to insure the equal distribution of heat to two or more tubes at the same time, they were placed in a beaker of water just kept at boiling temperature. Three solutions of safranin were used—one a '1 per cent.

1. *Bio-Chemical Journal*, Vol. II, p. 340.

solution and the others '15 per cent. and '2 per cent., and the number of drops of a suitable solution that was decolorised after heating for about half a minute was determined, and the glucose solution labelled accordingly. Equal quantities of urines were treated against these artificial solutions; thus the strength of glucose solution which exactly corresponded in its intensity of decolorisation and the time taken for the reaction with any given urine indicated the reducing power of that particular sample in terms of glucose.

When the urine did not quite correspond in reaction to any of the glucose solutions, the result midway between two solutions was taken; for instance a urine which gave a reaction a little more marked than a '11 per cent. solution of glucose and yet not quite so marked as a '12 per cent. solution, was taken as indicating the presence of carbohydrate matter equivalent to '115 per cent. glucose.

The following table indicates the results obtained in twenty normal urines.

No.	Specific gravity J	Before yeast (Total carbohydrate present)	After yeast (Unfermentable carbohydrate present)	Fermentable substance present
1.	1020	0'11 per cent.	0'055 per cent.	'055 per cent.
2.	1021	0'14 „	0'06 „	'08 „
3.	1015	0'10 „	0'04 „	'06 „
4.	1022	0'125 „	0'06 „	'065 „
5.	1025	0'11 „	0'05 „	'06 „
6.	1026	0'115 „	0'045 „	'07 „
7.	1025	0'10 „	0'035 „	'065 „
8.	1020	0'095 „	0'035 „	'06 „
9.	1014	0'08 „	0'035 „	'045 „
10.	1015	0'085 „	0'03 „	'055 „
11.	1023	0'10 „	0'045 „	'055 „
12.	1021	0'125 „	0'04 „	'085 „
13.	1015	0'085 „	0'03 „	'055 „
14.	1016	0'09 „	0'04 „	'05 „
15.	1018	0'11 „	0'06 „	'05 „
16.	1022	0'08 „	0'025 „	'055 „
17.	1025	0'135 „	0'045 „	'09 „
18.	1024	0'14 „	0'06 „	'08 „
19.	1024	0'10 „	0'05 „	'05 „
20.	1023	0'095 „	0'035 „	'06 „

Several hundred normal urines were examined, and it was found, as a general rule, that urine of average specific gravity from a healthy person contains on an average carbohydrate matter equivalent to from about .08 per cent to .1 per cent. glucose. A glance at the table indicates that the ratio of unfermentable to fermentable carbohydrate present is fairly constant, and varies roughly from 1 : 3 up to 1 : 2. In other words, from one-third to one-half of the total carbohydrate material in the urine is unfermentable carbohydrate. In many specimens the amount of fermentable sugar is in considerable excess of the unfermentable, though the limit is by no means great.

A normal urine, therefore, contains roughly carbohydrate to the equivalent of about 1 gramme to 1.5 grammes glucose per diem ; of this, from .33 gramme to .75 gramme is unfermentable carbohydrate material, probably largely iso-maltose, while the rest is probably glucose.

That glycuronic acid is not of much importance is indicated by the fact that removal of any traces of this compound does not seem to materially affect the relative proportions of the two groups ; this point, however, I intend to work out later on.

Several urines obtained from patients suffering from various diseases were examined from time to time. As a general rule it was found that the total carbohydrate varied but little, while the ratio of fermentable to unfermentable substance was generally much the same as in normal urine.

The following are the result of a few urines obtained from diseased patients.

No.	Specific gravity	Nature of case	Before fermentation (Total reducing carbohydrates)	After fermentation Total unfermentable carbohydrate	Total fermentable carbohydrate
1.	1028	Malignant disease of liver and colon	.115 per cent.	.05 per cent.	.065 per cent.
2.	1018	Pancreatic disease	.06 „	.025 „	.035 „
3.	1025	Malignant disease of the pylorus	.10 „	.055 „	.45 „
4.	1042	Diabetes... ..	6.5 „	.035 „	6.465 „
5.	1020	Chronic Bright's disease	.10 „	.035 „	.065 „
6.	1018	Pernicious anaemia	.090 „	.035 „	.055 „



Assuming, therefore, that the fermentable substance of ordinary urine is glucose, it would appear that ordinary urine contains roughly from '03 per cent. to '07 per cent. glucose. Such an amount agrees with the results obtained in testing by Fehling's solution for the creatinin present can easily prevent any reaction. In testing a urine that gives an ambiguous sugar reaction with other tests, safranin is exceedingly useful owing to the fact that ordinary 'interfering' substances, such as uric acid and creatinin, do not affect it. As a general rule it may be assumed that a urine which completely decolorises an equal quantity of a '25 per cent. to '3 per cent. safranin solution contains sugar in abnormal amount; in order to make certain whether the substance is glucose the urine ought to be fermented with yeast in an open flask at suitable temperature for twenty-four hours or so; after filtration, the contents of the flask should be again tested with safranin, and the diminution in reducing power noted. This excludes all possibility of interference by glycuronic acid and other substances, and gives an indication of the amount of sugar actually present. At the same time it is useful in a doubtful case in ascertaining whether the ambiguous reaction was due to pathological excess of sugar or merely physiological as the result of concentration. If the former is the case the ratio of fermentable to unfermentable substance may be high, say from 6 or 8 : 1 or even more; in the latter case the ratio will remain as usual—from 3 or 4 : 1 up to 2 : 1.

In all cases where urines give a doubtful reaction for sugar with such tests as Fehling's solution the writer has found the above plan very satisfactory and easy of application; the only disadvantage is that it takes at least a day, but at present there does not seem to be any very reliable and quick test for the detection of small amounts of sugar.

#### OTHER USES OF SAFRANIN

Safranin is therefore an excellent reagent for determining slight differences in the case of small amounts of sugar present in different solutions, or in the same solution at different times. In digestion

experiments the amount of starch changed can be comparatively easily obtained, while the differences in the carbohydrate contents of urine or blood, after standing for some time, as the result of glycolytic action, can be easily and fairly accurately determined. So far as my experiments go I have been unable to detect any appreciable reduction in the amount of sugar in urine after standing for some days, provided micro-organisms be excluded; it is intended, however, to work out these points with regard to blood and urine more fully later on. The above serves to indicate the uses of safranin for solutions containing small amounts of carbohydrate, and while the reagent possesses certain disadvantages with regard to its use as a routine test for the examination of urine in unskilled hands, it often proves exceedingly useful for the detection of traces, especially when combined with yeast fermentation.

#### CONCLUSIONS

1. Safranin is a general test for carbohydrate bodies of a certain type, and is one of the most suitable reagents for determining the presence of traces of carbohydrates in liquids; it is unaffected by all the ordinary 'interfering' substances of urine except those of a carbohydrate nature.

2. Safranin is not decolorised by albumin after long boiling, but the presence of albumin in the liquid to be tested interferes with the delicacy of the reaction; ammonia in excess acts in a somewhat similar manner.

3. By a combination of safranin and fermentation by yeast fermentable sugar can be easily demonstrated in every urine; along with the fermentable sugar, which is probably glucose, there is always present a definite amount of unfermentable carbohydrate.

The ratio of fermentable to unfermentable substances in normal urine varies roughly from 1 : 3 to 1 : 2; if the ratio is much disturbed the urine is probably pathological.

## THE ACTION OF METHYLENBLUE UPON COTTON FIBRE

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The nature of the staining act in respect of vegetable and animal fibres has not yet emerged from the region of discussion into that of ascertained fact. Owing to the desirability of making, in the light of recent research, more extended observations in this domain of enquiry, the present investigation of the amount of methylenblue taken up by cotton fibre in equilibrium with staining solutions of different concentrations was entered upon, the range of concentration employed extending over the most extreme range which the methods available permitted.

The only previous work on the staining of cotton fibre with methylenblue, which a careful search of the literature of dyeing has revealed to us, is that of Georgiewicz and Löwy (10), in 1895 (see Literature at the end of this paper). This work will be considered at some length later.

### METHOD

In this investigation the methylenblue employed was the double zinc salt,  $2\text{C}_{16}\text{H}_{18}\text{N}_3\text{SCl} + \text{ZnCl}_2 + \text{H}_2\text{O}$ , specially purified for injection *intra vitam*. An estimation of the nitrogen present in the dried dye gave 5.36 per cent. N, the theoretical amount being 5.45 per cent. N.

The cotton wool employed was absorbent cotton wool. Before use it was thoroughly washed in distilled water, the water removed

by centrifugalisation, and drying to constant weight effected at 100°C.

All estimations of methylenblue in cotton fibre or in solution were made by means of determinations, by Kjeldahl's method, of the amount of nitrogen present, the amount of dye being calculated therefrom. The amount of dye, which had to be estimated, was small in the case of dyed cotton fibre, nevertheless fairly concordant results were obtained as a glance at column 11, Table I, shows. Colorimetric methods were not available for the whole series of concentrations employed, and were only made use of for the weakest concentrations (Series D, Table I). The temperature of experiment was 44° to 46° C., throughout.

The method of procedure adopted in these experiments was the following. A weighed quantity of cotton wool was placed in a measured amount of a solution of methylenblue of known concentration, contained in a stoppered bottle kept in an incubator at 44° to 46° C. After a lapse of from two to thirty-one days, during which the mixture of cotton wool and staining solution was from time to time agitated, the dyed cotton was removed, care being taken to avoid cooling of the staining solution. The latter was quickly removed by strong centrifugalisation, after which the dyed cotton was weighed in the moist condition (column 7, Table I), and then dried to constant weight at 100° C. (column 6, Table I). After drying, the nitrogen in the dyed cotton wool was determined by a Kjeldahl estimation of the nitrogen it contained (column 8, Table I). A suitable amount of the staining solution was also taken at the end of the staining period and its content of methylenblue similarly determined by means of a Kjeldahl estimation (column 5, Table I). With the aid of the latter a correction, representing the dye contained in the small amount of staining solution mechanically adhering to the cotton fibre after centrifugalisation (column 9, Table I), was applied to the estimation of the total amount of methylenblue in the fibre, and in this way the true amount of dye taken up by the cotton ascertained (column 8, Table I).

In addition to the above method of estimating the percentage of methylenblue taken up by cotton fibre, the same may be determined



from the loss of concentration taking place in the staining solution (column 13, Table I), but such determinations, since they represent the difference between two estimations of methylenblue (except in Series D where colorimetric estimations were made) are less accurate than the direct estimation of the amount of dye in the fibre. Nevertheless they are given, for the sake of comparison, in Table I, but are omitted in Table II.

It may be observed that the total amount of dye in the cotton fibre could not be determined with a sufficient degree of accuracy by reference to the increase in weight of the dried cotton after dyeing.

#### EXPERIMENTAL WORK

The results obtained by the above method are given in Table I. It will be seen that the range of the initial concentration of the staining solution lies between 4.195 per cent. and .0046 per cent. It was

TABLE I. INTERACTION OF COTTON FIBRE AND METHYLENBLUE. TEMPERATURE 44° C. TO 46° C.

1	2	3	4	5	6	7	8	9	10	11	12	13
Series	No. of experiment	Duration of experiment	Concentration of methylenblue in staining solution :		Weight of dried cotton wool at end of experiment	Weight of dyed cotton wool before drying	Amount of dye in cotton wool at end of experiment, determined by Kjeldahl estimation of N	Amount of dye in staining solution adhering to cotton wool at end of experiment calculated from columns 5, 6 and 7	Calculation from results in columns 6, 8 and 9 of : amount of dye taken up by cotton wool	percentage of dye in cotton wool	Calculation from loss of concentration of dye in staining solution (columns 4, 5 and 6) of : amount of dye taken up by cotton wool	percentage of dye in cotton wool
			at beginning of experiment	at end of experiment								
A1	1	2 days	4.195 %	4.061 %	2.9821 g.	4.2821 g.	.0712 g.	.0528 g.	.0184 g.	.61 %	.0214 g.	.72 %
	2	2	4.195	4.061	3.0850	4.4031	.0733	.0535	.0202	.66	.0214	.70
	3	4	4.195	4.070	2.9516	4.2548	.0754	.0529	.0225	.77	.0200	.68
	4	4	4.195	4.052	2.9072	4.2472	.0765	.0545	.0220	.76	.0229	.79
A2	5	6	.454	.285	3.2917	4.9222	.0275	.0030	.0245	.75	.0286	.87
	6	6	.454	.295	2.8411	4.1195	.0255	.0028	.0227	.78	.0254	.90
	7	6	.454	.295	2.8683	4.1810	.0244	.0029	.0215	.75	.0254	.90
B2	8	4	—	.247	6.4126	9.2730	.0514	.0071	.0443	.69	—	—
	9	4	—	.218	4.6391	7.0401	.0353	.0059	.0294	.63	—	—
	10	4	—	.248	6.6699	9.7407	.0479	.0076	.0403	.61	—	—
	11	6	.0473	.0282	6.0896	14.5752	.0328	.0024	.0304	.45	.0286	.47
C1	12	6	.0473	.0276	5.5050	13.6167	.0320	.0022	.0298	.54	.0295	.54
	13	6	.0473	.0241	6.0231	14.4589	.0322	.0020	.0312	.52	.0348	.58
	14	6	.0473	.0266	5.8608	14.7947	.0345	.0034	.0311	.53	.0311	.53
	15	14	.0322	.0150	6.5878	9.8655	.0327	.0005	.0322	.49	.0273	.41
C2	16	14	.0322	.0157	5.5071	8.2608	.0319	.0004	.0315	.57	.0263	.47
	17	14	.0322	.0157	5.6312	8.4652	.0327	.0004	.0329	.57	.0263	.47
	18	14	.0322	.0150	6.5634	10.3054	.0353	.0005	.0349	.53	.0273	.42
	19	31	.0046	.0028	6.3391	9.7680	.0289	.0001	.0288	.45	.0287	.45
D	20	31	.0046	.0028	6.2158	9.3720	.0285	.0001	.0284	.46	.0281	.45
	21	31	.0046	.0024	6.2904	9.6573	.0344	.0001	.0343	.55	.0338	.53

determined to employ a series of concentrations forming a geometrical progression in which each member stood to the next member in the ratio of about 1 : 10, so that the total range of concentrations stood approximately in the ratio 1 : 10 : 10<sup>2</sup> : 10<sup>3</sup>. The duration of the period of staining presented some difficulty. Upon this point further observations are desirable, but it would appear, from a comparison of the results given in column 11, Table I, that equilibrium is attained by about the fourth day. The low result in Series A1, column 11, is presumably due to the staining period of two days being too short; on this account the mean of these two estimations is omitted in Table II. The probable error of the mean of the various series (cp. column 4, Table II) lies within .05 per cent. Nevertheless, this range of error is sufficiently large to affect noticeably the regularity of the variation in the estimated percentage of dye in the fibre in the different series, as a glance at Table II shows.

The percentage of methylenblue in the dyed fibre varies but little, ranging from .48 per cent. with the lowest concentration of staining solution to .765 per cent. with the highest. If an attempt is made to apply the formula

$$C = a C'^{\frac{1}{n}}$$

governing adsorption (see p. 449) it is found that the values of the two constants  $a = 1.20$  and  $\frac{1}{n} = .1$  agree fairly with the actual estimations as is shown in Table III, where the estimated (in brackets) and the calculated percentages of dye in cotton fibre, corresponding to the different concentrations of staining solution employed, are placed side by side.

TABLE II.—INTERACTION OF COTTON FIBRE AND METHYLENBLUE. MEAN RESULTS, TAKEN FROM TABLE I

Series	Duration of experiment	Concentration of staining solution at end of experiment	Concentration of dye in cotton wool, determined by estimation of N in dyed cotton wool
A2	... 4 days	... 4.061 %	... .765 %
B1	... 6 "	... .292	... .76
B2	... 4 "	... .240	... .64
C1	... 6 "	... .0266	... .51
C2	... 14 "	... .0153	... .54
D	... 31 "	... .0028	... .48

TABLE III.—CALCULATION, ON THE ASSUMPTION THAT  $a = 1.20$  AND  $\frac{1}{n} = .1$ , OF THE CONCENTRATION OF DYE IN COTTON WOOL IN EQUILIBRIUM WITH STAINING SOLUTIONS OF THE STRENGTH GIVEN IN TABLE II. THE FIGURES IN BRACKETS ARE THE MEAN CONCENTRATIONS GIVEN IN TABLE II.

Series		Concentration of staining solution at end of experiment	Concentration of dye in cotton fibre calculated from the formula : concentration of dye in cotton fibre = $\sqrt[1.0]{\frac{\text{concentration of dye in staining solution at end of experiment}}{1.20}}$		
A2	...	4.061 %	...	.76 %	(.765)
B1	...	.292	...	.72	(.76)
B2	...	.240	...	.72	(.64)
C1	...	.0266	...	.58	(.51)
C2	...	.0153	...	.53	(.54)
D	...	.0028	...	.49	(.48)

It will be observed that the value of  $\frac{1}{n}$  is smaller than that usually met with in adsorption experiments. The significance of this is considered in the next section.

### *The Nature of the Interaction of Fibre and Dye*

1. Before proceeding to interpret, in the light of recent researches, the results obtained in the preceding section, it will be necessary to consider the various processes which may conceivably occur when cotton fibre is placed in a watery solution of methylenblue, and also to refer briefly to the nature and the cardinal characters of such processes, so far as these are known.

The interaction may conceivably assume one or more of the following three types<sup>1</sup>: adsorption; chemical combination; the mutual action of oppositely charged bodies or particles.

2. By adsorption is meant the condensation of a dissolved substance upon a free surface.<sup>2</sup> In this condensation more energy is

1. Witt (19) suggested that in dyeing a solid solution of the dye in the fibre occurs. The distribution of the dye in fibre and staining solution does not, however, conform to the law of distribution between immiscible solvents (cp. Walker and Appleyard (18), p. 1335).

2. In such cases combination occurs in proportions which are not fixed, but depend on the concentration of the dissolved substance. For our knowledge of the quantitative relations obtaining in reactions of this type we are chiefly indebted to a series of monographs by v. Bemmelen (4), embodying his researches upon the hydrogels of ferric oxide, aluminium oxide, silicic oxide, stannic and metastannic oxides, manganic oxide, etc., and their combinations with water, hydrochloric acid, sodium chloride, barium hydroxide, etc. The subject has been studied from the microscopical standpoint by Bütschli (6), Quincke (16), and Hardy (13), and in respect also of electrical reactions by the latter author. The ultramicroscopical appearance of the solutions employed has been investigated by Zsigmondy and Liedentopf (20). Observations on the action of acids on silk fibre have been carried out quantitatively by Walker and Appleyard (18). The adsorption formula has recently been the subject of a monograph by Freundlich (8), in which further references to the literature will be found.



involved than that of simple passage from the gaseous to the liquid state.<sup>1</sup> Adsorption may take place at the surface of a simple solid, or at that presented by a gel or sol.<sup>2</sup> The distinctive characters of adsorption are: equilibrium between adsorbed and dissolved substance according to the relation

$$C = aC'^{\frac{1}{n}}$$

where  $C$  is the concentration of the adsorbed substance in the adsorbing body,  $C'$  is the concentration in the solvent of the substance used for adsorption, and  $a$  and  $\frac{1}{n}$  are constants; a limited range of values for  $\frac{1}{n}$ , varying between .5 and .8;<sup>3</sup> a lowering of tension at the solid-fluid surface, brought about by the substance used for adsorption. The last cannot be directly determined, the tension of the air-liquid surface being alone capable of measurement. As is seen in the Appendix to this article, methylenblue added to water causes an increase of the air-liquid surface tension.

3. Chemical action differs from adsorption in the circumstance that it is an interaction of atoms according to definite stoichiometrical ratios, while in adsorption molar action takes place. In consequence of this difference chemical action cannot be regarded as a limiting case of adsorption namely that in which the division of the adsorbing substance becomes progressively finer until at length it becomes molecular.<sup>4</sup>

In connection with the problem dealt with in this paper, the dissociation of chemical compounds is of especial interest. When salts in true solution dissociate, the relation

$$\frac{CaCb}{C} = K$$

holds, where  $Ca$ ,  $Cb$ , represent the respective concentrations of acid

1. The amount of heat given out when  $SO_2$  and  $NH_3$  are taken up by charcoal has been found by Berthelot to be greater than would be required to condense these substances from the gaseous to the liquid state at the temperature of experiment. The same occurs when water is taken up by the hydrogel of silicic acid.

2. The amount of a substance which is taken up by a hydrogel is influenced by the mode of preparation of the latter, by temperature and, to some extent also, in the case of reversible reactions, by a change from a more concentrated to a less concentrated medium, or *vice versa*. Fuller details are to be found in v. Bemmelen's published papers.

3. Freundlich (8), 1906, p. 71.

4. In true molar action it is to be expected that solutes of different types would be adsorbed indifferently. Selective adsorption is suggestive of interatomic action.

and base, resulting from hydrolysis of the salt, and  $K$  is a constant.<sup>1</sup> If the undissociated salt and one of the products into which it is dissociated are insoluble, the remaining dissociation product being soluble, then so long as the phase rule holds there is a critical concentration of the latter below which complete dissociation occurs, while above this concentration complete combination takes place.<sup>2</sup> If cotton fibre and methylenblue formed such a combination, then there would be a critical concentration of methylenblue below which all the dye would be discharged from the fibre, while above this concentration complete combination of dye and fibre would occur. It is, however, expressly pointed out by Gibbs (12) that the phase rule, in its ordinary form, is valid only when capillary actions and surfaces of small curvature are excluded. Whether it holds good for non-crystalloidal substances of enormously extended surface, such as are colloids, is doubtful. It may be that such a system would act in a different manner, and that its behaviour would resemble the dissociation of soluble crystalloids.<sup>3</sup> The investigation of such a type of dissociation, if it occurs, is not as yet practicable, since we have at present no means of measuring separately  $Ca$  and  $C$  in such heterogeneous systems. Nevertheless, the existence of solid chemical compounds of this type must be regarded as by no means excluded. If cotton fibre enters into a chemical combination with methylenblue<sup>4</sup> it must be of this type, since there is no critical concentration of methylenblue below which no combination occurs.

1. This is illustrated, for example, by solutions of potassium cyanide, ethyl acetate and urea hydrochloride in which, in consequence of hydrolysis, a condition of equilibrium is exhibited in which two reacting substances (potassium hydrate and hydrocyanic acid, ethyl alcohol and acetic acid, urea and hydrochloric acid) are combined in a varying degree, depending upon the relative concentrations obtaining. In homogeneous systems formed by solutions of salts derived from feeble acids or bases, such phenomena are common.

2. The behaviour of the combination of diphenylamin and picric acid, investigated by Walker and Appleyard (18), is an illustration in point. Both diphenylammonium picrate and diphenylamin are almost completely insoluble in water. In the dissociation of the former substance all the picric acid is given off below a critical concentration of this acid. The combined picric acid resembles, in this respect, water of crystallisation, which, as is well known, is only given up below a critical vapour tension.

3. This assumes, however, that in the solid compound of dye and fibre, the dye does not exist as a solution in the fibre.

4. The formula for cellulose is consistent with the assumption that it may be regarded as a free acid, its molecule containing a terminal  $\text{COOH}$  group.

4. Both the processes referred to in sections two and three may occur together. In such a case one may predominate and influence the aspect of the interaction accordingly, or again adsorption may take place at first and later chemical action be observed. The latter has, in fact, not unfrequently been noted in experiments on adsorption.

If cotton fibre is capable both of adsorbing methylenblue and of entering into a chemical combination with it for which the phase rule in its ordinary form does not apply, then the following chemical and physical actions may conceivably occur :—

- (a) Chemical action resulting in the formation of an undissociable compound ;
- (b) Chemical action resulting in the formation of a dissociable compound ;
- (c) Adsorption ;
- (d) Adsorption, together with chemical action, resulting in the formation of an undissociable compound ;
- (e) Adsorption, together with chemical action, resulting in the formation of a dissociable compound.

The following considerations will be of assistance in attempting to distinguish between these different cases.

In case *a*, in which an undissociable chemical compound is formed, no adsorption occurring, the amount of dye taken up would be fixed, and would be independent of the concentration of the dye in the staining solution ; here there would be no difficulty.

In case *b*, the amount of dye taken up by the cotton fibre might, if the phase rule did not apply to such a combination, vary with the concentration of the staining solution. In cases of this kind the adsorption formula could, no doubt, by choosing suitable values for the constants, be made to apply more or less closely to a series of determinations of equilibrial concentrations of dye, but then the value of  $\frac{1}{n}$  would not necessarily lie within the range obtaining in adsorption processes. In cases of chemical action, however, chemical affinity would be apparent, and the reacting substance would exhibit

selective action as to acid or basic dyes.<sup>1</sup> This criterion would fail, however, if the reacting substance were amphoteric in character.

In pure adsorption, case *c*, the distinctive character is the value of  $\frac{1}{n}$ , ranging between .5 and .8 (Freundlich).

The remaining two cases, *d* and *e*, would generally be characterised by the fact that the formula

$$C = aC'^{\frac{1}{n}}$$

would fit in more or less closely with the results obtained, but the value of  $\frac{1}{n}$  would lie outside the range met with in adsorption processes. In such cases selective action, if recognisable, would indicate chemical action.

In the case of cotton fibre no exclusive chemical character is exhibited. The fibre stains with methylenblue, but it also stains—and far more strongly—with acid dyes, *e.g.*, congo-red. After deep staining with methylenblue it decolourises completely in running tap water, under favourable circumstances, in about a week, but after staining with congo-red decoloration is slower, being not quite complete at the end of four weeks. These observations, therefore, being merely negative, do not aid in forming an opinion as to the likelihood of cotton fibre entering, or not, into chemical combination with methylenblue.<sup>2</sup>

1. The purely mechanical or rather physical explanation offered by the hypothesis of adsorption does not account for the fact that only a restricted number of dissolved substances are taken up. It is doubtful if, in such cases, adsorption alone exists. The following illustrations may be given :—v. Bemmelen (4), who observes (1900, p. 342): “Doch scheint es dass in manchen Fällen die Absorption sich hervorthut als ein Vorläufer der chemischen Verbindung,” found that the hydrogel of red manganese dioxide absorbs alkaline bases strongly, sulphuric acid and potassium sulphate slightly, and potassium chloride and nitrate not at all. Further, the hydrogel of silicic oxide takes up alkalies, including calcium hydroxide, more than acids; and the hydrogel of metastannic acid takes up hydrochloric acid strongly. The predominant or exclusive preference of fibres and tissue elements for so-called acid or basic dyes is another case in point. In a small number of cases it has been possible to ascertain that a chemical reaction takes place. v. Bemmelen observed that if hydrochloric acid is added to a hydrosol of ferric oxide, the particles of colloid collect together in the gel condition, forming a precipitate which takes up hydrochloric acid and from which ferric chloride is formed. Similarly, when barium hydroxide is added in sufficient amount to a hydrogel of silicic oxide, crystals of barium silicate having the composition  $\text{BaSiO}_3 \cdot 6\text{H}_2\text{O}$  make their appearance. Barratt (2) observed that methylenblue-eosin in alcoholic solution is decomposed in the staining act, the process not being attributable to preceding hydrolysis.

2. It is not improbable that cellulose  $(\text{C}_6\text{H}_{10}\text{O}_5)_n$  contains a carboxyl group (or more than one) in its molecule. A Kjeldahl estimation shows the presence of a small amount of N in absorbent cotton wool which has been thoroughly washed in distilled water. Cotton fibre arises by a saturation of the protoplasm of vegetable cells with cellulose. It is probable that some of the original proteid material remains attached to the cellulose molecule, which would then contain one or more  $\text{NH}_2$  groups giving it also a basic character. Owing to the large size of the cellulose molecule the presence of a single  $\text{NH}_2$  group would have little effect on the percentage composition of cellulose.



Under these circumstances the sole guiding factor is the value of  $\frac{1}{n}$ . If this lies within the range met with in adsorption processes, then the interaction may be regarded as an adsorption process. If the value of  $\frac{1}{n}$  lies outside this range, then the interaction must be regarded (cp. section 5) as a chemical combination attended, it may be, with an adsorption process as well. If the chemical compound formed by cotton fibre and methylenblue (assuming such to occur) is dissociated to a considerable extent, then no further analysis of the degree in which adsorption accompanies chemical combination is possible. If, however, extremely little dissociation occurs, within the limits investigated, then it is possible to determine approximately the amount of dye in chemical combination and the amount adsorbed, since, if the former were subtracted from the latter, the resulting values would represent the amount adsorbed and would conform to the adsorption formula, with a value of  $\frac{1}{n}$  lying within the limits obtaining in pure adsorption processes.

Applying these considerations to the results given in Table II, it was found, by making a series of trials, that, if the amount of dye in chemical combination is assumed to be '47 per cent., the percentages of adsorbed methylenblue become those given in column 2, Table IV, while the value of  $\frac{1}{n}$  becomes '67 and that of  $a$  4'545. It will thus be seen that, in order to obtain a value of  $\frac{1}{n}$  lying between the limits given by Freundlich, it is necessary to assume that the greater part of the methylenblue in the cotton is chemically combined and that only a relatively small fraction (especially so at the lower concentrations of staining solution) is adsorbed.

TABLE IV

Concentration of methylenblue in staining solution	Concentration of methylenblue in cotton wool	Concentration of methylenblue in cotton calculated on the assumption that $a = 4'545$ and $\frac{1}{n} = '67$
4'061 %	... [ $'76 - '47 =$ ] '29 %	... '35 %
'292	... [ $'72 - '47 =$ ] '25	... '16
'240	... [ $'72 - '47 =$ ] '25	... '14
'0266	... [ $'58 - '47 =$ ] '11	... '07
'0153	... [ $'53 - '47 =$ ] '06	... '06
'0028	... [ $'49 - '47 =$ ] '02	... '03

These figures, owing to the difficulty of making sufficiently delicate determinations of the amount of methylenblue in dyed cotton, over the range of concentrations studied, are necessarily merely approximate, but they serve to illustrate the mode in which it is suggested that the results given in Table I should be treated. It is interesting to note that if the amount of dye in chemical combination is assumed to be '47 per cent., then the molecular weight of cellulose would be about 70,000.<sup>1</sup>

5. In the interaction of cotton fibre and methylenblue in watery solution, there is no reason to suppose, that any process comparable to the mutual attraction of oppositely charged particles occurs, for a watery solution of methylenblue under the ultramicroscope is optically homogeneous (Michaelis, Biltz), and no decided movement of the dye in an electric field has been observed (Bayliss (3)).

6. Summarising the above we may say that the staining of cotton fibre by methylenblue in watery solution must, owing to the low value calculated for  $\frac{1}{n}$  in Table II, be viewed as a mixed process of chemical combination and adsorption, the former predominating.

7. Reference must here be made to the observations of Georgiewicz and Löwy (10) on the action of methylenblue upon mercerised cotton fibre. Their results are given in Table V, which is taken from their paper.

TABLE V.—INTERACTION OF MERCERISED COTTON FIBRE AND METHYLENBLUE AT 14° TO 17° C., OBSERVED BY GEORGIEWICZ AND LÖWY (*loc. cit.*)

No. of experiment	Concentration of staining solution at end of experiment		Concentration of dye in cotton wool		$\sqrt[3]{\frac{\text{concentration of dye in staining solution at end of experiment}}{\text{concentration of dye in cotton wool}}}$	
1	...	'0111 %	...	'61 %	...	'365 %
2	...	'0052	...	'48	...	'356
3	...	'0023	...	'38	...	'348
4	...	'0012	...	'30	...	'356
5	...	'0007	...	'25	...	'362
Mean						'357

1.  $\frac{\text{Molecular weight of methylenblue}}{\text{Molecular weight of cellulose}} = \frac{317}{126 \times n} = (\text{approximately}) \frac{.47}{100}$ . Therefore  $126n = \frac{31700}{.47} = 67,490$ . A terminal COOH group would confer an acid character upon the cellulose molecule.

These results show that in this case the action is an adsorption process in which  $a = \cdot 375$  and  $\frac{1}{n} = \frac{1}{3}$ . In mercerised cotton the action of alkali has obviously produced a modification of the cellulose molecule, causing it to lose its acid character and to be no longer capable of reacting chemically with the basic dye. The amount of adsorption is greater than that taking place in our experiments with unmodified cotton fibre, as is seen from the calculated percentages in Table VI. This difference indicates that when chemical combination of fibre and dye has occurred, the capacity of the fibre for adsorption of the dye is thereby lowered. It may be that in mercerised cotton an amount of alkali is in combination with the fibre corresponding to the methylenblue which, in our experiments, appears to be chemically combined. It is to be noted that in some later experiments with picric acid as staining agent for cotton and silk, a value of  $\frac{1}{n} = \cdot 1$  was obtained by Georgiewicz (11).

TABLE VI.—CALCULATION ACCORDING TO THE RESULTS OBTAINED BY GEORGIEWICZ AND LÖWY (TABLE V) OF CONCENTRATION OF DYE IN COTTON WOOL IN EQUILIBRIUM WITH STAINING SOLUTIONS OF THE END-STRENGTHS OBTAINING IN TABLE II.

Series	Concentration	Concentration of dye in cotton wool calculated from the formula :	
		concentration of dye in cotton wool =	$\sqrt[3]{\frac{\text{concentration of dye in staining solution at end of experiment}}{\cdot 357}}$
A2	... 4'061 %	... 4'47 %	
B1	... '292	... 1'86	
B2	... '240	... 1'74	
C1	... '0266	... '83	
C2	... '0153	... '67	
D	... '0028	... '39	

### SUMMARY

The action of methylenblue in watery solution upon cotton fibre is, owing to the low value of  $\frac{1}{n}$ , inconsistent with a pure adsorption process. It is best interpreted as a mixed process of chemical combination and adsorption, the former being the chief process.

## APPENDIX

*The air-fluid tension of an aqueous solution of methylenblue*

The following series of observations was made by Donnan's (7) method, T. 16.5° C.

Fluid in pipette			No. of drops	Sp. gr. of fluid	Relative surface tension
Distilled water	...	...	385	1.000	
"	...	...	387		
"	...	...	385		
"	...	...	385		
"	...	...	387		
"	...	...	387		
"	...	...	386		
			386 mean	...	100
Methylenblue 4 per cent. solution in distilled water	...	...	353	1.010	
"	...	...	352		
"	...	...	351		
"	...	...	350		
"	...	...	355		
"	...	...	351		
"	...	...	350		
			351.5 mean	...	110.9

It will be seen the surface (air-fluid) tension of water is increased 10.9 per cent. by the addition of 4 per cent. of methylenblue (= .126 M).



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## A CONTRIBUTION TO THE CHEMISTRY AND PHYSIOLOGICAL ACTION OF THE HUMIC ACIDS

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The physiological action of the humic acids on the living plant organism offers a field of work in which there is room for much investigation.

The present paper, which is preliminary to a larger contribution on the physiological effects of these acids on plant life generally, deals in the first place with the chemical composition of the substances hitherto known collectively under the general name 'Humic Acid.' In addition, in Part II, it is shown that it is possible to use the living organism as an auxiliary, which confirms and emphasizes the points of structural differences in these compounds which are indicated by chemical analysis.

This line of evidence, although seldom utilised by chemists, is of great value in determining minute differences of chemical composition in complex molecules of similar constitution, and is of special service in the case of the humic acids, to which the ordinary methods of organic chemistry cannot well be applied.

### PART I. CHEMICAL

The decomposition of complex carbohydrates and albumen by means of mineral acids to give a series of brown amorphous compounds, closely related to the natural humic acid, has engaged the attention of biological chemists for many years, and, until recently, it has been generally accepted that the acid obtained in this way

from cane sugar is identical in chemical structure with the natural acid, which may be extracted from peat or other decaying cellulosic tissues. This claim for identity rested largely on the fact that the general chemical properties of the acids derived from both natural and artificial sources are very similar, and, moreover, in certain cases, the composition of each as determined by elementary analyses, agrees within the limits of accuracy admissible in such work. Both acids are amorphous, sparingly soluble, brown solids, which dissolve easily in caustic alkalis to give highly coloured solutions from which the free acid may be precipitated by means of hydrogen chloride.

Experiments which are at present in progress here on the physiological effect of humic acid on plant cultures suggested that the supposed identity of the acids in question should be subjected to further proof, and accordingly specimens of natural and artificial humic acid were prepared by different methods, and critically compared.

It has already been noted<sup>1</sup> that, when prepared by the decomposition of ammonium humate, the acid contains combined nitrogen, and we therefore prepared the natural acid from heather peat by two methods; in one series of preparations the ammonium salt was precipitated with hydrochloric acid, and in parallel experiments the potassium salt was similarly treated.

The artificial acid was prepared from pure cane sugar by the action of hydrochloric acid, and in this case also the compound was obtained through the medium of both the potassium and the ammonium salt.

It is well known<sup>2</sup> that the nature of the 'humic acid' obtained from cane sugar varies greatly with the method of preparation, the solubility of the product showing great variation according to the concentration and nature of the acid used. We accordingly adopted conditions which gave a product displaying in appearance, solubility, and general properties the closest resemblance to the natural acid. We thus obtained four distinct yet apparently identical specimens

1. Eggertz, *Centralblatt*, 89, 343; André, *Comptes Rendus*, 127, 414; Festini, *Centralblatt*, 1902, 182.  
2. Conrad and Guthzeit, *Berichte*, 19, 2849.

of humic acid (two from natural and two from artificial sources), and the composition of each as obtained in several different preparations was compared.

In addition to estimations of carbon, hydrogen and nitrogen, the methoxyl content ( $\text{CH}_3\text{O}$ ) was also determined by Zeisel's process in order to test the suggestion that humic acid contains alkyloxy groups. The results show that considerable uniformity exists in the composition of the different specimens of the acid derived from cane sugar, and that although the acid obtained through the ammonium salt contains a small amount of combined nitrogen, the analytical composition is identical with that obtained from the potassium salt. The natural acids, however, invariably contained considerably less carbon, showing that little reliance is to be placed on the superficial identity of the compounds.

The average compositions of the different specimens deduced from a series of concordant combustions are compared in the following table :—

			C	H	O	N
Artificial	{ Through potassium salt	...	64.70	4.55	30.71	Nil
	{ Through ammonium salt	...	64.74	4.69	29.81	0.76
Natural	{ Through potassium salt	...	56.67	5.16	35.68	2.49
	{ Through ammonium salt	...	54.29	4.94	38.16	2.61

It will be seen that considerable difference exists in the composition of the two specimens of the natural acid, and this is partly accounted for by the somewhat variable nitrogen content of each. A factor of greater importance, however, is the different amount of inorganic impurities invariably present in the two cases. After combustion of the natural acids a quantity of mineral ash containing aluminium and ferric oxides was left behind in the boat, and, although this was allowed for in calculating the results, yet in the absence of any information concerning the form in which this extraneous matter existed in the specimens, it is possible that the analyses might be vitiated to a slight extent by its presence.



A more striking illustration of the different chemical structure of the natural and artificial acids is furnished by the results of the Zeisel determinations. It was found that the former compounds never contained more than from 1.71 to 2.47 per cent. of alkyloxy groups, calculating the result as  $\text{CH}_3\text{O}$ , whilst the artificial acid gave a much higher result, namely, 6.47 per cent. As the compounds resinified with hydriodic acid our results must only be regarded as approximately accurate, but if, as is generally supposed, the molecule of humic acid contains at least twenty-four carbon and twelve oxygen atoms, then in the natural acid the percentage of methoxyl found does not correspond to even one molecular proportion, whilst in the artificial product we must assume the existence of at least one such group. [ $\text{C}_{23}\text{H}_{21}\text{O}_{11}$  ( $\text{CH}_3\text{O}$ ), requires  $\text{CH}_3\text{O} = 6.15$  per cent.]

Further evidence of a similar nature was furnished by the analyses of the barium salts obtained from the different acids. This method, in fact, affords a much better index of identity or difference than analyses of the acids themselves. Thus the two hypothetical acids,  $\text{C}_{20}\text{H}_{16}\text{O}_{10}$  and  $\text{C}_{30}\text{H}_{28}\text{O}_{15}$ , would give, on combustion, figures which are almost identical, and certainly within the limits of experimental error. Assuming each to be a dibasic acid, their barium salts would show a difference of about 7 per cent. in the barium content, and the values for carbon and hydrogen would also be affected. It will be seen from the table of analyses of the various barium humates described in the experimental part that the identity of the salts of the artificial acids is confirmed, and that the natural barium humates differ in marked fashion from them and from each other.

Our results also throw some light on the problem of the empirical formula of humic acid. In spite of the fact that no crystalline derivatives of the acid are known, investigators have not hesitated to speculate as to the probable complexity of the molecule, and in Lippmann's *Chemie der Zuckerarten* (Vol. II, p. 1245) an excellent summary is given of various possible formulae which have been ascribed to the compound by Mulder, Stein, Berthelot, André and others.

We have been able to test these formulae, which naturally show great variation by application of the analytical results we have obtained.

The best agreement so far as the analyses of the artificial free acids is concerned is shown with Mulder's formula,  $C_{40}H_{30}O_{15}$ ,<sup>1</sup> and also with that ascribed to the compound by Berthelot and André,  $C_{18}H_{16}O_7$ .<sup>2</sup> The latter formula is, however, inadmissible when the analyses of the barium humate are taken into consideration, as, assuming the acid to be dibasic, the barium content of the salt would be as high as 28.6 per cent. If, however, the formula be somewhat modified and altered so as to express a molecule of double complexity, then the analyses of both the acid and its salts agree with the calculated figures. Thus the formula  $C_{39}H_{32}O_{14}$  requires  $C=64.64$ ,  $H=4.42$ , and assuming the barium salt to have the composition  $C_{39}H_{30}O_{14}Ba$  the calculated percentage of barium is 15.98. The mean of several combustions of the artificial acid gave  $C=64.70$ ,  $H=4.55$ , while the barium content of the salts varied between 15.31 and 15.65 per cent., a close agreement considering the nature of the compounds.

It is, of course, more difficult to speculate as to the composition of the natural acid, as the problem is here complicated by the presence of combined nitrogen and traces of inorganic impurity. Our analyses agree, however, approximately with the formula of the acid obtained from Dopplerite,<sup>3</sup>  $C_{24}H_{28}O_{14}$ , although the carbon content is somewhat higher. A barium salt of the composition  $C_{24}H_{22}BaO_{12}$  has already been described by Demel,<sup>4</sup> and it has been suggested that the corresponding acid  $C_{24}H_{24}O_{12}$  is a double anhydride derived from the acid in Dopplerite. It should be pointed out here, however, that estimations of barium in natural humates should be accepted with caution, as the inorganic impurities contaminate the ignited residue, and hence increase the apparent barium content. In our experiments the ignited residue was extracted with hydrochloric acid, and the barium estimated by precipitation with sulphuric acid. In this way a result was obtained which was invariably 3 or 4 per

1. *Journal für praktische Chemie*, I, 21, 203; 32, 331.

2. *Comptes Rendus*, 112, 916, 1237.

3. Mayer, *Die landwirtschaftliche Versuchsstationen*, 29, 313.

4. *Monatshefte für Chemie*, 3, 763.

cent. lower than that found by merely igniting the residue with sulphuric acid. Disregarding the combined nitrogen, the analyses of the natural acid obtained through the potassium salt stands between the values calculated for the Dopplerite acid, and its anhydride.

$C_{24}H_{28}O_{14}$  requires:—

$$C = 53.33, H = 5.18$$

$C_{24}H_{24}O_{12}$  requires:—

$$C = 57.14, H = 4.76$$

$$\text{Found } \dots C = 56.67, H = 5.16$$

The barium salt agrees most closely with the second formula—

$C_{24}H_{22}O_{12}Ba$  requires:—

$$C = 45.07, H = 3.44, Ba = 21.44$$

$$\text{Found } \dots C = 46.87, H = 3.27, Ba = 21.60$$

If we take into account the combined nitrogen present, then the analytical figures agree with the anhydro acid in which one oxygen atom is replaced by nitrogen. Thus  $C_{24}H_{24}O_{11}N$  contains 2.79 per cent. N, the value actually found being 2.49 per cent.

With regard to the natural acid obtained through the ammonium humate the low barium content of the salt indicates a greater molecular complexity. The hypothetical acid  $C_{30}H_{30}O_{17}$  and the corresponding salt  $C_{30}H_{28}O_{17}Ba$  require respectively:—

$$\text{Acid } \dots \dots C = 54.38 \quad H = 4.53$$

$$\text{Found } \dots \dots C = 54.29 \quad H = 4.94$$

$$\text{Salt } \dots \dots C = 45.17 \quad H = 3.51 \quad Ba = 17.20$$

$$\text{Found } \dots \dots C = 45.31 \quad H = 3.76 \quad Ba = 17.53$$

The amount of combined nitrogen present in the acid is accounted for by the modified formula  $C_{30}H_{30}O_{16}N$ , for which the theoretical values for carbon and hydrogen are nearly the same; the calculated nitrogen content in this case is 2.12 per cent., the value found being 2.61 per cent.

## EXPERIMENTAL

*Preparation of Natural Humic Acid from Peat*

The usual method of preparation was followed, the well-dried peat being finely divided and thoroughly washed to remove soluble salts. The filtered product was then digested in quantities of 100 grammes with 1500 c.c. water containing 66 grammes hydrogen chloride.<sup>1</sup> The residue was then digested with excess of 5 per cent. ammonium hydrate, and the humic acid precipitated by the addition of a slight excess of strong hydrochloric acid to the filtrate.

The amorphous brown precipitate was filtered on a Büchner funnel, washed with water, and dried first on porous plates and afterwards in a vacuum over sulphuric acid. The average yield is about 4 grammes dry acid from 100 grammes peat.

In a parallel series of preparations a solution of 1 per cent. potassium carbonate was used in place of ammonium hydrate in the above process, the average yields being thereby increased to 5 grammes.

The extensive dilution of the various solutions used in these preparations is rendered necessary by the difficulties experienced in the various filtrations. The effect of alkalis on the cellulosic tissues of the peat is to produce gelatinous syrupy liquids, which filter extremely slowly, even when a vacuum of 3 mm. is used. In very dilute solutions, however, the filtration proceeded more readily, but was nevertheless slow.

*Preparation of Artificial Humic Acid from Cane Sugar*

We obtained artificial humic acid displaying the closest physical resemblance to the natural compound by boiling a solution containing 200 grammes cane sugar dissolved in 367 c.c. water and 133 c.c. strong hydrochloric acid. The reaction was continued under an inverted condenser for thirty hours, the precipitated solid being filtered off every ten hours. The dark mass thus obtained was finely powdered,

1. *Comptes Rendus*, 1898, 127, 414.



digested repeatedly with hot water, and then extracted with 5 per cent. potassium carbonate (or ammonium hydrate), the humic acid being precipitated and purified exactly as in the previous case. Yield 12 grammes.

All attempts to obtain the acids in a crystalline or colourless state failed. Very little colouring matter was extracted when specimens were boiled with alcohol, acetone, acetic acid or ethyl acetate; and when the alkaline salts were boiled for several days with animal charcoal no decolorisation was effected.

#### *Comparison of the Natural and Artificial Acids*

In appearance, solubility, and behaviour towards alkalis the acids appear identical. The solubility in water is very slight, and in the case of the natural acid only amounts to 0.0076 at 20°. Both acids give sparingly soluble calcium, barium or magnesium salts.

The natural acid even when purified by re-precipitation gives a small quantity of mineral ash on ignition, but no residue was obtained from the artificial form. The results of the analyses which are summarised in the theoretical part show that the artificial acids obtained through the medium of the potassium and ammonium salts are practically identical in composition, save that the latter contains a small percentage of combined nitrogen. The natural acids are distinguished by their lower carbon content, higher percentage of hydrogen, and also by the presence of an estimable amount of methoxyl and combined nitrogen. Here again the acid obtained through the ammonium salt contained most nitrogen.

We endeavoured to supplement the above evidence, distinguishing between the natural and artificial acids, by determinations of the specific rotations of each. On account of the extreme dilution of the aqueous solutions the observed readings were very small, and our results are, therefore, only to be accepted qualitatively. Both acids are strongly dextro-rotatory, and so also are their potassium salts. In the latter case also very dilute solutions were used on account of the intense brown colour of the compounds. The artificial acids appeared to be distinctly more active than the natural forms, but

it is possible that the activity of the former may have been partly due to the presence of traces of glucose derived from the decomposition of the cane sugar.

It has already been shewn by Conrad and Guthzeit<sup>1</sup> that fructose is completely decomposed by boiling with 5 per cent. hydrochloric acid, but that quantities of unaltered glucose remained after prolonged boiling with even 20 per cent. acid.

As already explained in the introduction the comparison of the various preparations of the acids was extended by examination of the barium salts derived from each. These were prepared in each case by the addition of excess of barium chloride to solutions of the alkaline salts of the acids. A weighed quantity of acid derived from the ammonium salt was neutralised by the addition of very dilute ammonium hydrate, and precipitated by means of barium chloride. In the case of acids prepared through the potassium salt the method of preparation was similar, save that caustic potash replaced ammonium hydrate. The filtered salt was well washed with water, and dried in a vacuum until constant in weight. Several duplicate preparations were made and analysed. The results tabulated below, which contain the average figures obtained in several concordant analyses, confirm our opinion that the artificial acids are identical, but differ in marked fashion from the natural compounds.

#### ANALYSES OF BARIUM HUMATES

	Method of preparation :—	Artificial	Natural		
		From ammonium humate	From potassium humate	From ammonium humate	From potassium humate
C	... ..	52.66 %	52.43 %	45.31 %	46.87 %
H	... ..	3.56	3.35	3.76	3.27
Ba	... ..	15.31	15.34	17.53	21.60

The difference in composition shewn in the salts of the natural acids, together with the fact that the acids themselves possess different carbon contents, seems to indicate that the composition of the natural

1. *Berichte* 19, 2509, 2575.

acid varies greatly with the method of preparation, according to whether the potassium or ammonium salt method is used. This result emphasises in marked fashion the structural difference which must exist between the artificial and natural acids, as in the former case the barium salts obtained by both methods are identical in analytical composition.

## PART II. PHYSIOLOGICAL

To determine the action of the acids and their salts on the living organism various cultures are in progress both with autotrophic and heterotrophic flowering and flowerless plants. Of these experiments only certain series will be here referred to, in which the effect of acids and salts has been determined on fungi, because these give the nutritive values of the humic compounds for such organisms, and also because they to a certain extent corroborate the points of difference referred to in the analytical part of this paper. We endeavoured to determine the nutritive value of the acids and their salts as carbon and nitrogen compounds respectively, and from the results to draw conclusions as to the relative carbon and nitrogen content of the four series of acids and salts. Artificial cultures were prepared with the usual precautions to secure purity. The acids in these were in concentrated solutions. The salts specially selected were the potassium salts, as their ready solubility allowed of a considerably greater range of concentration than in other cases. In addition we are of the opinion, although we have not yet verified the point, that the potassium salt is the form in which these compounds are presented to plants under natural conditions. In the cultures the potassium salts were used in concentrations varying from 0.0125 to 0.6 per cent. Some of the cultures were in liquid media, to others a sufficient amount of gelatine was added to produce a solid medium for convenience in handling.

In the series to determine the carbon values, the carbon was supplied in the form of the concentrated solutions of the acids or their potassium salts at different concentrations the nitrogen in the mineral form of potassium nitrate, or organic nitrogen as peptone.

The corresponding controls were potassium nitrate + glucose and peptone + glucose.

In determining the nitrogen values the nitrogen was supplied as 0.15 per cent. of the potassium salts, the carbon as glucose, as against controls of peptone + glucose and potassium nitrate + glucose. In addition to these substances the usual ash constituents were added to the culture media. As a concentrated aqueous solution of the free acid contained only 0.07 gramme per litre of the compound, and we used relatively weak solutions of the potassium salts, it was necessary to equalise the conditions by using proportional amounts of the corresponding nitrogen or carbon compounds in the controls. In these the amount of peptone was 0.7 and of glucose 0.01 per cent.

Pending the completion of investigations into the cardinal points of temperature in relation to nutrition with the acids and their salts, the series of cultures here referred to were carried out at ordinary room temperature, and no very special allowance was made for differences of the osmotic pressure of the media. As a matter of fact, we had evidence which appeared to indicate that the optimum nutritive temperature is higher for the artificial than for the natural acids, and this point was not altogether ignored in judging of results. Further, certain differences in the growth habit of our plants were put down to differences in the osmotic pressure of the media, on the analogy of Beauverie's researches<sup>1</sup> with mineral solutions on moulds. The analogy, however, was not forced, in view of Laurent's observations<sup>2</sup> that an increase in the concentration of the medium if of organic materials is more readily endured by the plant than if the medium be of mineral matter only.

That we did not more fully allow for the effect of the temperature and pressure factors may be held as militating to a certain extent against the validity of our results. As against this may be placed the fact that we used as our experimental plant *Penicillium glaucum*, which is well known not only to have an extensive range of nutritive

1. *Comptes Rendus*, Vol. CXXXII, p. 226.

2. *Comptes Rendus*, 1902, Vol. CXXXV, p. 871.



materials but also to have marked accommodatory powers to differences of temperature and of osmotic pressure. Further, we aimed here at obtaining not absolute quantitative but relative qualitative results as to whether the acids and their salts could or could not act as carbon and nitrogen food substances respectively. Again, ordinary room temperature is a nearer approximation to the conditions under which the humic substances, the natural compounds especially, act on plants in nature than, say, an artificial temperature of  $31^{\circ}\text{C}$ ., the maximum nutritive temperature for *Penicillium* on sugar.

In determining the nutritive values of our acids and salts as carbon compounds we were precluded in the absence of accurate temperature data, as above mentioned, from utilising as criteria the 'economic coefficients.' After all, this proved to be no disadvantage. We started with the assumption, afterwards shown to be correct, that glucose + peptone had the highest nutritive value, and that the acid + peptone must have a lower coefficient, which, of course, would vary with the position of the optimum nutritive temperature in the scale. We found evidence to show that not only did the optimum for glucose differ from that of the acids, but that the acids themselves had different nutritive optimum temperatures: the relative order of superiority being glucose, artificial acids, natural acids. As there exists this difference in the optima, and as the coefficients are affected by change of temperature, by variations in the respiratory activity consequent on change of temperature, and further, as the pressure of the culture medium also changes with temperature and is not without effect on growth, it is obvious that whether the comparative nutritive values are based on the coefficient at the different optima or on other data from cultures grown at a uniform temperature the results are in neither case absolute owing to the experimental error due to the disturbing factors introduced by variations in the respiratory activity or of the osmotic pressure respectively.

We generally found that the initial growth was most rapid in the natural compounds, next in the artificial, while the glucose controls were usually the last to start. Had we estimated the carbon

values in the usual way by the relative rapidity of growth in the earlier stages, we should have put the natural compounds first, and the others in the above order of nutritive values. What actually occurred was that after the initial retardation the cultures in the artificial compounds and glucose controls made up on the others subsequently, and not unusually surpassed them, producing in a given time colonies of larger size. We compromised matters by noting the average rate of growth, and the ultimate size attained by the colonies after the lapse of a given time.

For convenience in reference the following symbols are used :—

Natural Humic Acid (from peat)—

Prepared through the ammonium salt ... ..  $N_a$

„ „ potassium salt ... ..  $N_k$

Artificial Humic Acid (from sugar)—

Prepared through the ammonium salt ... ..  $A_a$

„ „ potassium salt ... ..  $A_k$

The letter K prefixed to either of the above symbols indicates the potassium salt of the acid in question thus :—

The Potassium Salt of the Natural Humic Acid—

Prepared through the ammonium salt ... ..  $KN_a$

„ „ potassium salt ... ..  $KN_k$

The Potassium Salt of the Artificial Humic Acid—

Prepared through the ammonium salt ... ..  $KA_a$

„ „ potassium salt ... ..  $KA_k$

In order to emphasise the distinction between the natural and artificial acids, brought out not only by chemical analysis but also by the different physiological action of the acids on the living organism, we suggest that the name humic acid be applied only to the acid as prepared from peat, while the artificial acid prepared from sugar should be known by the new term of *saccharo-humic acid*.

#### *Nutritive Values of the Acids and Salts as Carbon Compounds*

These values were based on the results of the following series of cultures :—

*Series I.*—The nitrogen was supplied in the mineral form of potassium nitrate, the

carbon in the organic form of saturated solution of  $N_a$ ,  $N_k$ , and  $A_a$ . (In the latter case, as already noted in the analytical part, traces of glucose are possibly present in addition.)

*Series II.*—The acids were replaced by  $KN_a$ ,  $KN_k$  and  $KA_a$  of concentrations 0.0125, 0.025 and 0.05 per cent. The controls were potassium nitrate + glucose.

*Series III.*—The nitrogen was supplied in the organic form as peptone, carbon as saturated solutions of  $N_a$ ,  $N_k$  and  $A_k$ .

*Series IV.*—For the acids were substituted  $KN_a$ ,  $KN_k$ ,  $KA_a$  of concentrations 0.025, 0.05, 0.075 per cent.

*Series V* contained peptone and  $KN_a$ ,  $KN_k$ ,  $KA_a$  and  $KA_k$  of concentrations 0.1, 0.15, 0.3 and 0.35 per cent. Peptone + glucose formed the controls to III, IV and V.

The general conclusions drawn from the results of these cultures are that, contrary to the experience of Nikitinsky,<sup>1</sup> not only the artificial but also the natural acids as well as their potassium salts serve as sources of organic carbon for *Penicillium*. As evidenced by differences in its growth forms and histological characters, the mould can discriminate between the artificial and the natural acids; the nutritive value varies with the character of the nitrogenous food supply; there is a differential value dependent on the concentration and the chemical nature of the acids and salts, and possibly also on the osmotic pressure of the medium.

When the results of the various cultures are reviewed seriatim the following conclusions are arrived at:—

*Series I.*—The nutritive combination potassium nitrate + acid is much inferior to nitrate + glucose: not only in its effects on vegetative growth, but also in conidial production; the conidia in the former never develop their characteristic colours. As carbon sources, the natural acids combined with mineral nitrogen are better than the artificial acids. The order of value is  $N_a$ ,  $N_k$ ,  $A_a$ .

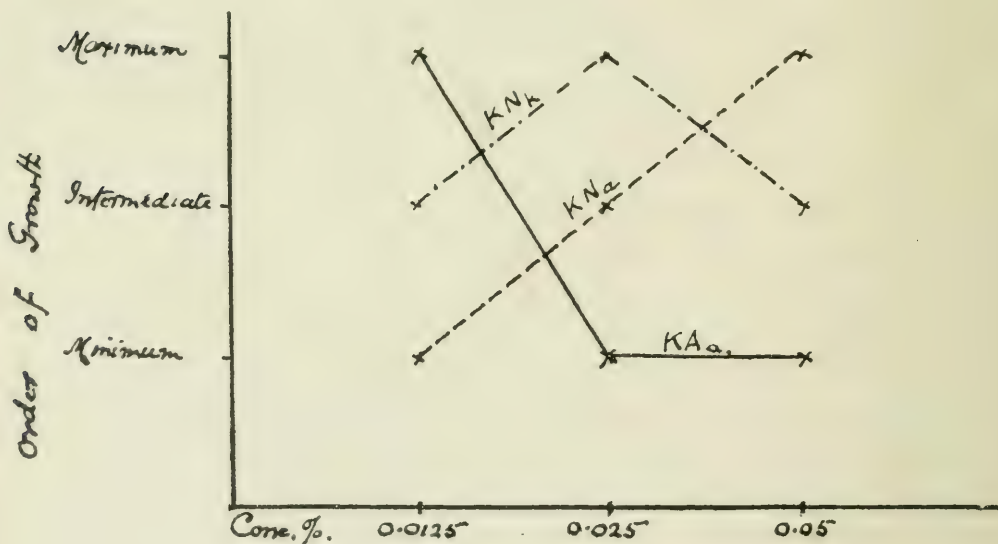
This is exactly the opposite of what might be expected from the carbon contents of the acids, the artificial compound having the highest carbon content. On the other hand, the natural acids are richest in nitrogen, and this additional nitrogen may have had its effect on growth. Besides, the factor of osmotic pressure may enter here also, that of the artificial compounds being presumably

1. *Jahrb. wiss. Bot.*, 1902, Bd. XXXVII, p. 385.

lower than that of the natural acids. This difference in pressure may be inferred from the habit of growth in the various cultures, the mycelia grown in the natural acid showing most submerged growth, while that in the artificial compound shows least. Beauverie,<sup>1</sup> in experiments with mineral cultures on moulds, showed that increased concentration was accompanied by a greater amount of submerged growth of the mycelium.

*Series II* gave, on the whole, similar results to *Series I*. While in both series the growth was very scanty even in cultures several weeks old, that in the nitrate + salts was, if anything, even slightly poorer than in nitrate + acid, and *a fortiori* much less than the control.

The variation in growth with varying concentration of the salts is expressed in the following curves :—

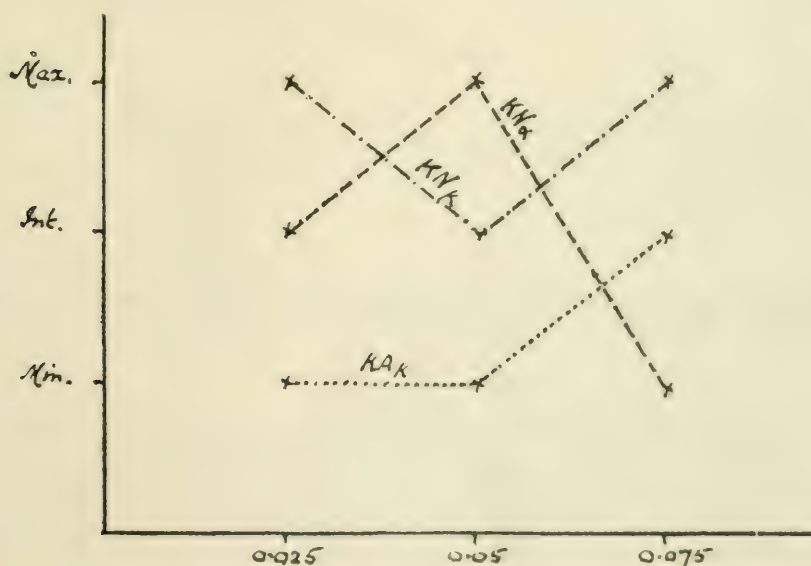


At low concentration the artificial acid gave best results ; at higher concentration, however, the natural acid yielded the maximum growth.

1. *Comptes Rendus*, Vol. CXXXII, p. 226.

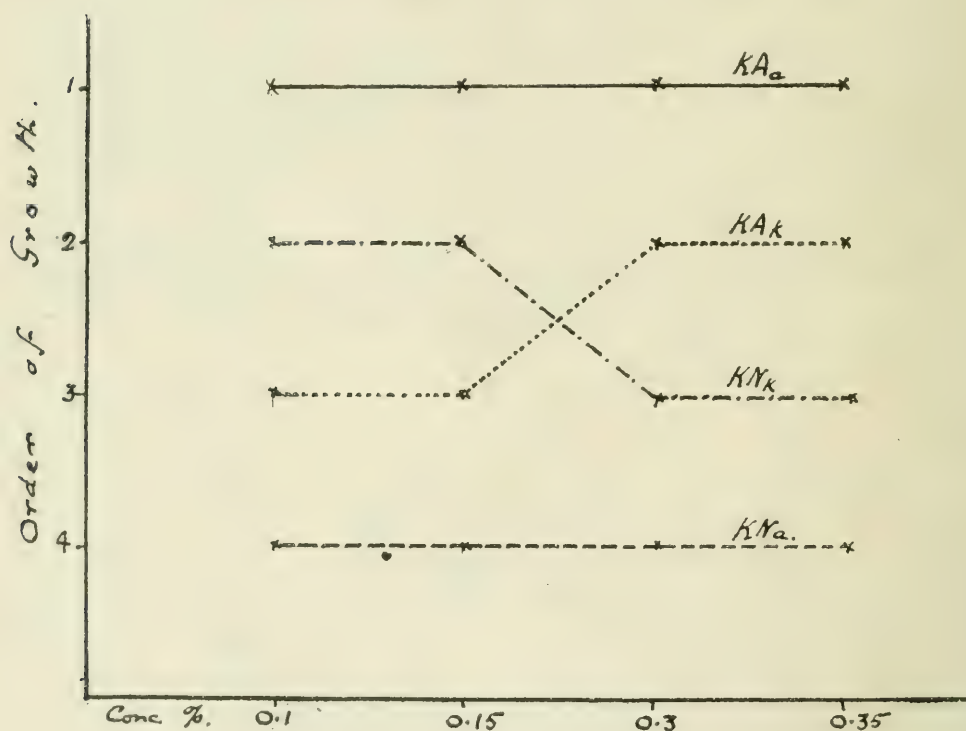


In Series III, IV, and V the nitrogen was supplied as peptone, the carbon as the acid in concentrated solution or as the potassium salts of different degrees of concentration. This formed a very much better nutritive medium than either I or II; the plants grew almost as well as in the control, peptone + glucose, there being good vegetative growth, conidial formation being normal and the spores developing their characteristic colour. Presumably the carbon available in the peptone was drawn upon, but that the acids and salts exerted their influence was obvious from a comparison of the resulting growths. In Series III, peptone + acids, the artificial acids gave best growth, the order being  $A_k$ ,  $N_a$ ,  $N_k$ . The cultures in the glucose controls and in the artificial acids were late in commencing growth, but ultimately surpassed those in the natural acids, the resulting colonies being much larger in size (Plate I, figs. 1, 2, 3, and 4). Comparison of the subjoined curves illustrating the growth in Series IV (peptone + salts) with those of Series II (nitrate + salts) shows that for the same degree of concentration 0.025 and 0.05 per cent. the relative gradation of growth is similar in both series; at higher concentration, 0.075 per cent., the salts of the acid prepared through the potassium salts— $KN_k$  and  $KA_k$ —lead.



Of the three salts in the above,  $\text{KN}_k$  and  $\text{KA}_k$  contain the most carbon, and the evidence of this is seen in the rise of their respective curves at concentration above .05 per cent., the  $\text{KN}_a$ , which has least, falling to third place.

In Series V the salts of the four acids were used at still higher concentration combined with peptone, and these series gave the most satisfactory comparative results of all. The growth was good, although somewhat inferior to that of peptone + acid and *a fortiori* to that of the control.



The following points are brought out in the above curves; above 0.3 per cent. the potassium saccharo-humates have highest value as carbon food compounds, the  $\text{KA}_a$  leading throughout from 0.1 to 0.35 per cent. Of the two potassium humates the  $\text{KN}_k$  leads

all through. This corresponds exactly to the relative carbon contents of the four compounds as expressed on p. 466, the order being  $A_a$ ,  $A_k$ ,  $N_k$ ,  $N_a$ . Summarising the results as to carbon values of the acids and their salts, we arrive at the following conclusions. The acids and their salts in combination with organic nitrogen serve as good nutrient carbon material, but less so than sugar + peptone; that the acids and salts do act as carbon supply, and that the carbon is not derived entirely from the peptone, is proved by the fact that the combination nitrate + acid or salt also acts as a food material, although of a very much poorer character than peptone + acid or salt.

The nutritive values vary with concentration, chemical character of the acids, and perhaps also with temperature. At and above 0.3 per cent. the saccharo-humates have highest carbon value, at lower concentration there is some variability.

*The Nutritive Value of the Humates and Saccharo-Humates as Nitrogen Compounds*

This was ascertained by series of cultures (Series VI) in which the carbon was supplied in the organic form as glucose, the nitrogen as 0.15 per cent. humates and saccharo-humates,  $KN_a$ ,  $KN_k$ ,  $KA_k$ ,  $KA_a$ —the mineral constituents were added, with the exception, of course, of nitrate. The controls were peptone + glucose. The order of growth here was  $KN_a$ ,  $KN_k$ ,  $KA_k$ ,  $KA_a$ . Plate II shows the appearance of ten days' old cultures. In the humates (Figs 1 and 2) there was a luxuriant growth; in the saccharo-humates (Figs. 3 and 4) poor; some growth was, of course, to be expected in the  $KA_a$ , but an almost equal development of colonies occurred in the  $KA_k$ . When, however, the very great difficulty of keeping cultures quite nitrogen-free, and, further, the suspicion that *Penicillium* may have a slight power of assimilating free nitrogen is remembered, this growth is not inexplicable. The contrast however in the two sets of cultures is a very marked one, and can only be interpreted as due to the very much higher nitrogen content of the humate as contrasted with the saccharo-humate.

Contrasting the results of Series VI with the controls and the Series I-V, we draw the following conclusions. The combination humate + glucose has almost as high a nutritive value as peptone + glucose. Its nutritive value is greater than peptone combined either with the acids or with the humates or saccharo-humates, and *a fortiori* is better than nitrate + acids or salts, or even than nitrate + glucose.

The nutritive value of the acids and their salts as carbon and nitrogen compounds, respectively, for *Penicillium* may be tabulated as follows :—

Source of Nitrogen		Source of Carbon		Value
Potassium nitrate	...	Conc. N or A	...	Very slight
"	"	KN or KA ('0125-'05)	...	" "
"	"	Glucose	...	Fair
Peptone	...	Conc. N or A	...	High
"	...	KN or KA ('025-'35)	...	"
"	...	Glucose	...	Very high
KN	...	Glucose	...	" "

#### *Comparative Histology of the Mycelia in the different Cultures*

Microscopic examination of the mycelia grown in media containing humic and saccharo-humic compounds, respectively, confirms the view that these substances are not identical.

To refer here only to the characters of the vegetative mycelium we find that the hyphae grown in humic solutions exhibit the following points of contrast to those cultivated in saccharo-humic media. The former are more robust, septation is more frequent, and consequently the hyphal cells are shorter. At the same time the diameter of the cells is greater, while the cell walls are markedly thicker and generally of a brown colour, especially in the  $N_k$  cultures.

The branching is different in the two types. In the saccharo-humic cultures the branches were given off at an acute angle, while those in the humic cultures formed a right angle with the main hyphae.

In the manner of branching, in the delicate transparent character



of its hyphae, the mycelium of the saccharo-humic cultures closely resembled that from the glucose cultures, while the mycelium of the humic cultures by its thicker brown-walled hyphae of shorter cells was distinctly marked off from either of the others.

While holding that these differences indicate and are to be associated with differences in the chemical character of the humic and saccharo-humic compounds acting as food material to the mycelium, we, of course, admit the possibility of difference in osmotic concentration affecting the results to a certain extent. This has been already noted in reference to the relative amounts of submerged and floating mycelium in the fluid cultures containing the same percentages of the various compounds.

Laurent<sup>1</sup> has described for higher plants histological changes, such as increase in cell-wall thickness, shortening and widening of the cells and so forth, caused by differences in concentration of nutrient solutions containing organic substances. These differences are analogous to what we have found in the mycelia from our cultures containing equivalent percentages of the different compounds.

#### *Correlation of Physiological and Chemical Results*

The chemical investigations have shown that the artificial acids are richest in carbon, the relative carbon content is expressed in the descending series  $A_a$ ,  $A_k$ ,  $N_k$ ,  $N_a$ .

Comparing this series with the growth series as given in Cultures V we find that at 0.3 and 0.35 per cent. the relative nutrient value of the potassium humates and saccharo-humates is according to the order— $KA_a$ ,  $KA_k$ ,  $KN_k$ ,  $KN_a$ . That is to say there is here complete concordance between the chemical and physiological series, and the latter therefore supply auxiliary evidence of the accuracy of the chemical analysis.

This is further borne out by the results of Series III represented in Plate I. Here the growth in the artificial compound stands first. The photographs 2, 3 and 4 may be taken as a series of auxanograms

1. *Comptes Rendus*, 1902, 135, 871.

of the relative carbon content of the acids. The resemblance between figs. 1 and 2, the glucose and artificial acid cultures, respectively, is striking, and seems to support the suggestion already put forward that traces of glucose occur in the specimens of the artificial acids. We endeavoured to test this hypothesis further by yeast cultures in nutrient solutions where the concentrated solutions of the acids were substituted for glucose. From all the cultures traces of  $\text{CO}_2$  were given off, appreciably greater in the artificial forms, especially in  $A_k$ .

The iodoform test gave evidence of slight traces of alcohol in all the cultures. We, therefore, cannot regard this experiment as decisive of the question, for Nikitinsky (*loc. cit.*) has shown that certain bacteria can decompose humic acid with formation of  $\text{CO}_2$ , and it is possible that yeast has the same faculty, and that the acids themselves were undergoing decomposition; on the other hand, there is the possibility that the cultures although carefully prepared were not above suspicion as to purity. The point is being further investigated.

Somewhat better, though not quite conclusive, are our experiments to determine bio-chemically the presence of iron in the natural acids. In nutrient solutions in which the humates and saccharo-humates were substituted for iron salts, various aquatic plants were placed. Up to the present these appear to be going in the right direction, particularly some experiments with *Lemna*. As *Lemna* is an iron-storer we adopt the precaution of removing each old leaf as soon as a new one appears, and thus exhaust the original store of iron in the plant, which then has to depend on the nutrient solution for its supply. So far the experiments lend support to the chemical statement that only the natural acids contain iron. A subsidiary feature not without significance is that micro-algae develop freely in the cultures with the natural humic salts, but not in those containing the artificial salts.

The relative nitrogen content of the acids, as chemically determined, is expressed by the series (highest first)  $N_n$ ,  $N_k$ ,  $A_n$  ( $A_k$  none).

The sixth series of cultures gives a growth series agreeing exactly

with this. In Plate II, which is a series of photographs of the relative growth, we have auxanograms of the nitrogen content. Here the influence of the relatively large percentage of nitrogen in the natural acids (figs. 1 and 2) is evidenced by the very much greater growth, the small development in the artificial forms being correlated with the smaller nitrogen content of the compounds.

In conclusion, we have to express to Professor T. Purdie our great indebtedness, both for his courtesy in placing the ample resources of his Research Laboratory at our disposal for carrying through the chemical part of this research, and also for his advice so kindly given during the progress of the work.

#### SYNOPSIS OF RESULTS

1. Natural humic acid obtained from peat varies greatly in composition, according to the method of preparation. When prepared through the ammonium salt the percentage of carbon is lower, and that of nitrogen is higher, than when prepared from the potassium salt.

2. Natural humic acid contains more combined nitrogen and less methoxyl than the artificial forms.

3. The form of humic acid obtained from cane sugar, which resembles most closely the natural acid, differs greatly in chemical structure from the latter.

4. The artificial acid is unaltered in composition by conversion into the ammonium salt and reprecipitation.

5. Humic and saccharo-humic acid and their potassium salts are utilisable by *Penicillium* as sources of organic carbon.

6. Potassium humate and, to a less extent, potassium saccharo-humate serve as organic nitrogen food compounds for *Penicillium*.

7. The conclusions drawn from the physiological results of experimental cultures of *Penicillium* in the acids and their potassium salts are in concordance with those arrived at as the result of chemical analysis of the compounds.

## EXPLANATION OF PLATES

Plate I. A series of photographs of Petri Dish Cultures of *Penicillium* in

Peptone + glucose (fig. 1)

Peptone +  $A_a$  (fig. 2)

Peptone +  $N_a$  (fig. 3)

Peptone +  $N_k$  (fig. 4)

These represent auxanograms of the relative nutritive carbon values of glucose, the saccharo-humic and humic acids respectively.

Plate II. A series of photographs of Petri Dish Cultures of *Penicillium* in

$KN_a$  + glucose (fig. 1)

$KN_k$  + glucose (fig. 2)

$KA_a$  + glucose (fig. 3)

$KA_k$  + glucose (fig. 4)

These are auxanograms of the relative nutritive nitrogen values of the potassium humates and saccharo-humates respectively.





1



2



3



4

R. A. R. photo.

PLATE I. AUXANOGRAMS OF CARBON NUTRITIVE VALUES OF HUMIC AND  
SACCHAROHUMIC ACIDS AND CONTROL GLUCOSE





1



2



3



4

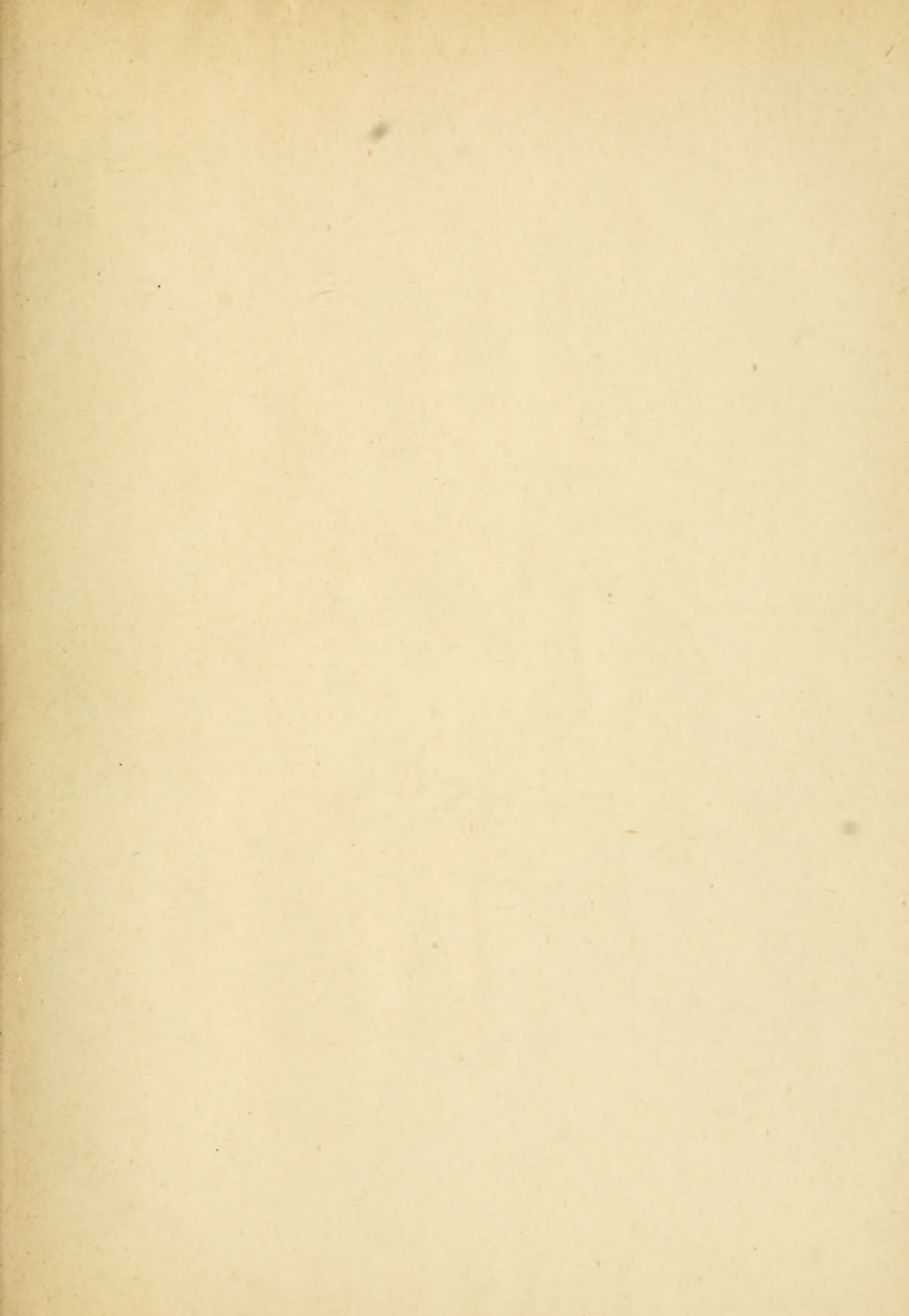
PLATE II. AUXANOGRAMS OF NITROGEN NUTRITIVE VALUES OF  
0.15% POTASSIUM HUMATES AND SACCHAROHUMATES















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